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THE VAGINAL SMEAR—BASAL BODY TEMPERATURE TECHNIC AND ITS APPLICATION TO THE STUDY OF FUNCTIONAL STERILITY IN WOMEN[1]

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THE MENSTRUAL FLOW is the most striking and obvious of the periodically recurring events during the child bearing years of a woman's life. Its occurrence is the result of changes in the uterus, detailed descriptions of which may be found in the literature cited at the end of this paper (1). The flow itself marks the end of a cycle of changes in the uterus.

The uterine cycle is not the only one to reflect the fluctuations of gonad function. The vaginal mucosa undergoes comparable cyclical changes which are readily amenable to study by the vaginal smear technic. The cycle of gonadal function is also reflected in the metabolic rate and basal body temperatures (2), the changes in the breasts (3, 4), in weight (5, 6, 7) and in bioelectric potentials (8, 9). In this report we propose to present in detail our studies using vaginal smears and basal body temperatures as they are related to gonadal function.

The Gonad Cycle

The cycle of ovarian changes begins with the singling out of one of the immature follicles to undergo a process of growth and maturation. As the follicle grows, its lining membrane secretes the hormone, estrone, which stimulates proliferation in the uterus and vagina. As the follicle matures some of its lining cells change their function and form lutein cells which are so named because they appear yellow. They are the forerunners of the corpus luteum whose full development occurs after ovulation. The lutein cells secrete the hormone, progesterone. The preovulative production of progesterone starts the secretory phase in the uterine and vaginal mucosa. After ovulation the space previously occupied by the follicle is rapidly filled by the multiplication of lutein cells. The lutein cells produce not only progesterone, but also estrone. Since estrone causes proliferation of the uterine and vaginal mucosa, proliferation continues throughout the period of corpus luteum function. Progesterone causes secretion in the uterus and vagina. These changes are the precursors of the uterine development in pregnancy. Thus, every cycle of an adult woman is designed as preparation for pregnancy. Pregnancy, however, is not the subject of this study. When pregnancy fails to occur, the corpus luteum regresses. Consequently, there is lessened pro-

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duction of the hormones necessary for continued growth of the uterus and vagina. With the stimulus to continued growth gone, the uterine blood supply diminishes. Ischemia leads to necrosis of the endometrium which is subsequently expelled. This is the menstrual flow. The cycle of gonad function may therefore be summarized as follows.

(*a*) A follicle develops, producing estrone.

(*b*) At maturity, just before rupture, lutein tissue appears which produces progesterone.

(*c*) After ovulation the corpus luteum develops rapidly; progesterone production increases proportionately. Estrone production continues.

(*d*) The corpus luteum begins to atrophy. The reason for atrophy of the corpus luteum is still unknown but thought to be due to inhibition of luteinizing hormone production of the pituitary gland. Production of both estrone and progesterone regresses to the vanishing point.

(*e*) A new follicle starts developing and the cycle is repeated.

The Vaginal Cycle

The vaginal cycle is the result of the gonadal cycle. Estrone leads to the proliferation of the vaginal mucosa just as it does in the case of the uterine mucosa. As the vaginal mucous membrane increases in thickness its superficial cells grow farther and farther away from their blood supply and undergo the same essentially degenerative process (cornification) that occurs in the skin. Consequently in smears made from the superficial vaginal lining the progress of the proliferative process may be estimated by the degree of cornification seen in the smears. When only moderate proliferation occurs, the smears consist of squamous epithelial cells with large vesicular nuclei and granular cytoplasm. As proliferation progresses the superficial cells seen in the smears show gradually increasing pyknosis of the nuclei and gradual disappearance of the granules in their cytoplasm. Pyknosis, a degenerative process, involves condensation of nuclear material; the large vesicular nuclei become small and dense. The granular cytoplasm is replaced by keratin, the horny material which is the chief constituent of our skin, hair and nails. As a consequence of further proliferation there is progressive cornification of the cells; the nuclei fragment and may finally disappear. Thus completely cornified cells, an evidence of maximum proliferation, are anuclear and take characteristic keratin stains (Plate I).

The smears are taken from the posterior wall of the posterior fornix of the vagina. This is a reliable site not commonly subject to friction and fairly uniform in its responses to hormone. A loop is inserted through a speculum into the posterior fornix. The superficial cells are gently scraped up and spread on a clean glass slide which is immediately dropped into fixative consisting of equal parts of alcohol and ether. The hydration and staining technic are described in detail by Papanicolaou (10). It is not necessary to dehydrate after staining or to mount in balsam unless the smear is to be kept as a permanent record. Ordinarily, it is adequate merely to blot the stained smear and examine under the microscope.

Upon examination it is immediately evident that on the slide there are not only epithelial cells but also varying amounts of bacteria, leucocytes, red blood cells and mucus. In our description of the cyclical changes in the vaginal smears we shall refer to fluctuations and changes in all these elements. It should be noted that the most important elements in the smear are the epithelial cells since these most accurately reflect the gonadal hormone production.

We present at this time a detailed description of the varieties or types of epithelial cells that will be found in the smears. For convenience these are classified into 8 types; it must be remembered that since cornification is a continuous process, intermediate types of cells will almost always be found.

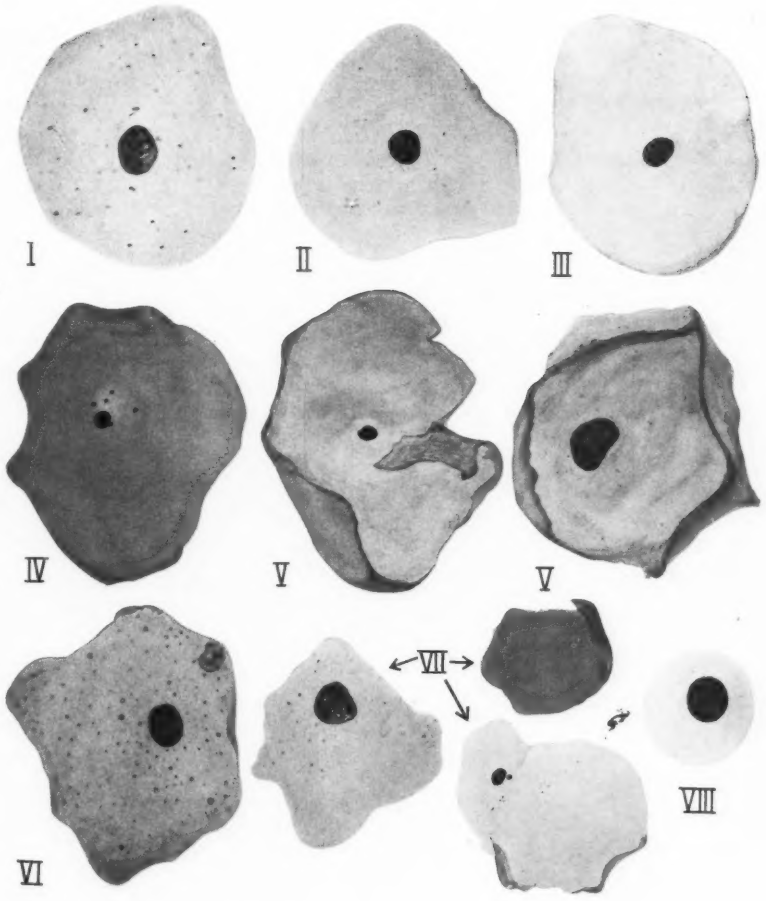

PLATE I. TYPES OF VAGINAL EPITHELIAL CELLS FOUND IN VAGINAL SMEARS. These represent only types described in the classification. Intermediate types are also always present but should easily be recognized as such.

Basal cells. Type VIII.[3] The vaginal mucosa of children, or old women, and of certain amenorrheic adult women is very thin and consists of basal layers of cells. These basal cells are characteristically round cells, somewhat larger in size than the large mononuclear leucocytes. Their nuclei are round, vesicular and approximately half the cell area. They are frequently in one or another phase of cell division. Their cytoplasm is somewhat granular and basophilic. In the normal adult woman basal cells are rarely seen, since the vaginal mucosa is usually several layers thick. Supra-basal cells are all squamous epithelial cells. In the nonpregnant woman they usually show various degrees of cornification. For convenience they may be classified into the following 7 types.

Type I. This cell type is about 2 to 3 times as large as the basal cell. Its cytoplasm is granular and stains either purple with hematoxylin or bluish with water blue. Its nucleus is about the same size as the nucleus of the basal cell; consequently, it is proportionately smaller. The nucleus is round and vesicular, but rarely in process of mitosis.

Type II. In Type II cells, minimal degenerative changes are seen, i.e. the cytoplasm is either pale lavender or very light pink. The nucleus begins to shrink, is somewhat denser than the nucleus in Type I, but still distinctly purple and somewhat vesicular. The nucleus is usually oval and purple in color. The differences between Types I and II are slight. They are sometimes difficult to distinguish. In general, the small nucleus, usually oval, and the comparatively smooth cytoplasm make the distinction possible.

Type III. Type III shows still further degeneration. The nucleus is again smaller and denser than Type II. The cytoplasm is characteristically free from granules and stains with eosin. The differences between Types II and III are small, but distinctive: the nucleus is denser and smaller; the cytoplasm is smooth and clear.

Type IV. The Type IV cells are completely cornified. The cytoplasm is without granules, completely keratinized and stained intensely with eosin. The nucleus is either extremely small and dense, or fragmented, or entirely absent. The nucleus or its fragments stain nearly black. The chief distinction between III and IV is therefore in the complete pyknosis of the nucleus.

Type V. Just as the process of cornification may be used to follow the degree of estrone production of the maturing follicle; so the process of desquamation, and the degeneration of the desquamated cells, their folding and vacuolization may be used as an indicator of the presence of progesterone. Progesterone stimulates secretion and counteracts the action of estrone. Type V cells have characteristically wrinkled cytoplasm, frequently with scalloped edges. Type V cells are usually clumped together or aggregated. The cytoplasm is usually free from granules and eosin-staining. The nucleus may be absent; it is most frequently pyknotic, but occasionally may be somewhat vesicular.

Type VI. Type VI cells are found associated with Type V, particularly after the desquamation of the cornified cells. These are cells which resemble Type II in that their nuclei are large and usually oval. They resemble Type V since their cytoplasm is characteristically wrinkled and their edges are scalloped. Their cytoplasm is occasionally granular and occasionally vacuolated. These are younger cells than the Type V cells, but are just as significant of progesterone activity.

Type VII. About a week before the onset of the next menstrual flow, the corpus luteum begins to regress. Since the corpus luteum produces both estrone and progesterone, its regression means progressive diminution in the production of both

[3] Basal cells were classified as Type VIII instead of Type I because the other cell types had already been found, described, and their descriptions used in teaching before these basal cells were described.

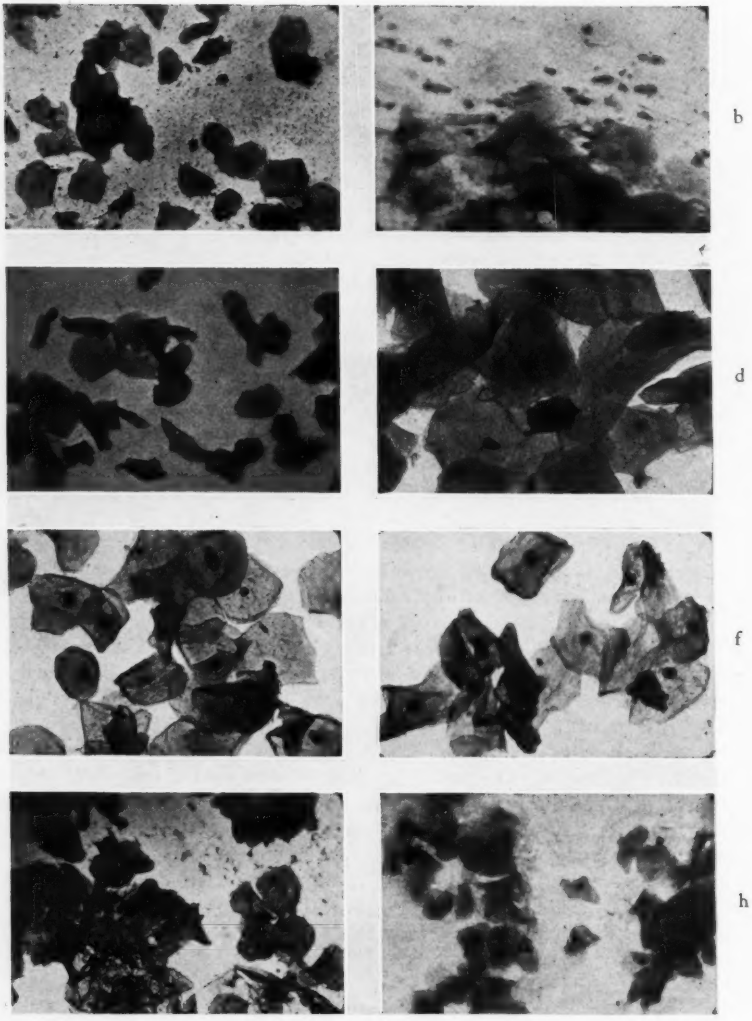

PLATE II

(See next page for legend)

hormones. The mucosa of the vagina, therefore, is progressively less stimulated and also regresses. The cells which had proliferated under the influence of estrone slough off and fragment. Type VII cells therefore are really fragmented cells. The nuclei may be vesicular or pyknotic. About the nucleus there may be only a wisp of cytoplasm, pale lavender in color; or the cells may be broken into approximately equal halves, only one part containing a nucleus. The cytoplasm or rather cytoplasmic fragments may be partially cornified or granular. Vacuoles may be present. The only distinctive feature about Type VII cells is their broken-up condition.

In the event of pregnancy the corpus luteum does not regress. It increases in size and produces increasing amounts of both estrone and progesterone. In response to the increasing amounts of hormone new cell types appear which may be used to diagnose pregnancy. Since, however, this is not the subject of the present communication, the description will be reserved for the future.

We shall now present descriptions of typical smears taken at various phases of the cycle. For convenience we shall start with the postmenstrual phase of the cycle.

Postmenstrual phase. At this time the follicle is just beginning to develop. Estrone production is slight, progesterone production negligible; consequently proliferation of the vaginal mucosa is just beginning and the epithelial cells in the smear are predominantly of the Döderlein type. There are also many leucocytes and some thick mucus (Plate II a).

Preovulative phase. After the immature follicle develops towards maturity it secretes progressively increasing amounts of estrone. The estrone stimulates proliferation of the vaginal mucosa. The mucosa grows thicker. Its superficial cells become more and more cornified. Therefore, the smears of the preovulative phase of the cycle show epithelial cells which are progressively more cornified; i.e. Types II and III; followed by Types II, III, and IV; then Types III and IV, and finally IV predominates. During this phase of the cycle the numbers of bacteria and leucocytes diminish progressively. The mucus becomes more abundant but more watery and stains progressively less until it cannot be seen in the smear at all (Plate II b).

As the time of ovulation approaches, the follicle, now nearly mature, begins to secrete minimal amounts of progesterone, the presence of which, even before ovulation, is immediately reflected in the smear by the appearance of Type V cells and by increased desquamation of the Type IV cells. Desquamation is the sloughing of the superficial layers of the vaginal mucosa. It is probably due to the fact that progesterone stimulates secretion in the vaginal mucosa as in the uterine mucosa (Plate II c).

PLATE II. Vaginal Smears during the normal menstrual cycle. (Papanicolaou stain.)

a. Oc. 7.5 Obj. 16; 80×. Post-menstrual smear, cell Types I and II, indicative of an early estrogen effect.
b. Oc. 7.5 Obj. 8; 160×. Post-menstrual smear, cell Types II and III, with an occasional Type I. Note presence of leukocytes in mucous strands. This smear indicates some increasing estrogen effect.
c. Oc. 7.5 Obj. 16; 80×. Pre-ovulative smear, cell Types II, III, and IV, indicative of a high level of estrogen.
d. Oc. 7.5 Obj. 8; 160×. Ovulative smear. Cell Types III and IV, indicate nearly maximal estrogen effect on vaginal mucosa.
e. Oc. 7.5 Obj. 8; 160×. Post-ovulative smear. Cell Types III, IV as above, and Type V, indicates minimal progesterone action—the typical folding and curling of the cell edges.
f. Oc. 7.5 Obj. 8; 160×. Smear of luteal phase. Cell Types V and VI, indicate prolonged progesterone action. The folding and curling are present even in these less cornified cells from deeper vaginal layers.
g. Oc. 7.5 Obj. 16; 80×. Same as above. Note the extensive desquamation and aggregation indicating the action of both estrogen and progesterone on the vaginal mucosa. Note also that leukocytes are present.
h. Oc. 7.5 Obj. 16; 80×. Pre-menstrual smear. Cell Types V, VI, VII and I. The presence of Type VII, cell debris is typical of the premenstrual phase; the smear is "dirty," the cell and nuclear outlines are indistinct although nuclear size and granularity of cytoplasm indicate that these cells are frequently from deep layers of the vaginal mucosa.

Ovulative phase. When the follicle matures it normally ruptures. There is a temporary diminution in hormone production which may be reflected in the vaginal smear by the appearance of a few or many red blood cells. At ovulation, there is a marked desquamation of cell Types III and IV with a few more Type V cells than were seen in the latest preovulative smears. There is usually no recognizable mucus and very few leucocytes and bacteria. The ovulative smear can usually be recognized even without staining because the cells appear in flat plaques like shingles. The ovulative smear is most readily recognized by contrast with the immediately postovulative smear (Plate II d, e).

Postovulative phase. The postovulative phase is the phase of the rapidly developing corpus luteum. The corpus luteum produces both estrone and progesterone; consequently proliferation and secretion are stimulated. This is reflected in the vaginal smears by continued desquamation of cells which are characteristically clumped and folded together. The cells are Types IV and V with occasional Type VI's. There is a marked influx of leucocytes which is not so characteristic as the leucocytosis in rat smears. More and more bacteria are also present. The mucus remains abundant, but becomes thicker and consequently again visible in the smears (Plate II f).

Luteal phase. As the corpus luteum develops to its maximum function, progesterone becomes the dominant hormone. The stimulus to secretion is most obvious while further proliferation is less evident. Desquamation is faster than proliferation. This is reflected in the smears by the appearance of reversal of the process of cornification. The vaginal smears continue to show desquamation and aggregation, but the nuclei are progressively larger and more vesicular and the cytoplasm progressively less keratinized, i.e. the Type V cells now look like the clumped Type II or III cells (Plate II g). The smears continue to contain abundant mucus, leucocytes and bacteria.

When the corpus luteum begins to regress, hormone production diminishes. The vaginal mucous membrane, therefore, becomes still thinner. The nourishment of the superficial cells is no longer maintained. They begin to fragment. Consequently during this phase of the cycle Type VII cells begin to appear and grow progressively more frequent as regression of the corpus luteum progresses.

Premenstrual phase. The premenstrual phase is a phase of low hormone level. Occasionally the very beginnings of follicle development are present with their slight stimulus to proliferation; but usually this phase is characterized by a very thin vaginal mucosa, consisting of only two or three layers of cells. These cells are therefore Types VII and I with an occasional Type II. A few red blood cells may be present. Leucocytes and mucus are abundant. Bacteria are somewhat less abundant than in the postmenstrual phase. It is very difficult to distinguish the premenstrual phase from the postmenstrual phase since both are usually periods of low hormone level (Plate II h).

Menstrual phase. The period of menstrual flow like the pre- and postmenstrual periods is a period of low hormone production. The vaginal mucosa is very thin. Since the menstrual flow usually consists in large part of blood and mucus, the vaginal smear during the period of menstrual flow is dominated by the presence of abundant red blood cells and of quantities of mucus. The epithelial cells reflect the earliest phases of follicle development and are therefore predominantly Types I and II. A few Type VIII cells are frequently present. In rare cases follicle development may progress to a high level; the epithelial cells may then be Types II and III. The epithelial cells are ordinarily tangled in mucus, may be somewhat degenerated (with ragged edges or indistinct nuclei giving the impression of Type VII cells).

In the event that menstruation fails to occur because of pregnancy, the typical

premenstrual and menstrual smears which are indicative of a low hormone level are replaced by smears indicative of a high hormone level. These smears are not necessarily characteristic for pregnancy but do indicate the persistence of a functional corpus luteum. They are characterized by the persistence of Type V and VI cells and by increasing secretion or mucus and leucocytes. Occasionally a few red blood cells may be present and may represent the implantation bleeding of lower primates. The persistence of a smear indicative of corpus luteum function at a time when premenstrual or menstrual smears are expected should make the investigator suspicious that pregnancy may have occurred.

In presenting the vaginal smear method as a very useful means of estimating gonadal function, it is, of course, necessary to note the limitations of the method and possible errors which may result from its careless use. In the first place just as a single blood sugar determination cannot be used to diagnose diabetes without knowing a great deal about the patient, so a single cornified smear cannot be used to diagnose ovulation. This is especially true since there is evidence that chronic irritation due to infection, masturbation, or drugs (11) can produce cornified smears in the absence of normal gonad function. Consequently the vaginal smear method should not be relied upon when there is vaginal infection or irritation. It should be noted that usually the vaginal smear and basal rectal temperatures will show a discrepancy when some nongonadal influence affects one or the other, thus the combination of the two methods avoids in large part the unreliability of either method alone.

Associated with the changes in the vaginal mucosa are changes in the acidity of the vaginal secretions. The vaginal secretions are usually acid (pH 4.5 to 5.0). During the menstrual flow the expelled uterine secretions are mixed with those of the vagina, and reduce the acidity to near the neutral point (pH 6.7 to 7.0). It has been found by Séguy and Simmonnet (12) that at the time of follicle maturity the cervical glands secrete large quantities of a faintly alkaline fluid (pH 7.2). This secretion temporarily reduces the acidity of the vaginal content during the ovulative phase of the cycle. Thus the curve of vaginal pH shows two peaks, the one corresponding to the period of menstrual flow, the other to the period of follicle maturity and ovulation (13).

Another change which histologists have described in the vaginal mucosa is the cyclical appearance and disappearance of glycogen. This appears in the vaginal mucosa during the proliferative phase of the cycle. Its function there has not been fully explained. It disappears with the sloughing of the vaginal mucosa in the premenstrual period. The presence of glycogen may account for the presence of the typical vaginal flora (*bacillus Döderleini*) which requires glycogen as a nutrient medium. The presence of this flora and of a high degree of acidity in the vagina may therefore be considered evidence for an intact and normally functioning vaginal mucous membrane. Indeed, clinical observation confirms this. The presence of a pathogenic infection is incompatible with the high degree of acidity of the normal vagina. Thus the rationale for local estrone application to an infected vaginal mucous membrane is to induce proliferation, acid production, glycogen deposit, and a medium favorable to acid-forming bacteria and therefore unfavorable to the usual pathogenic bacteria.

Temperature cycle. In previous communications (2, 14) the cycle of basal, rectal temperatures was shown to be related to the cycle of gonadal activity. When suitable precautions are used, the temperature curve is a most useful tool in the study of the human sex cycle, particularly when correlated with vaginal smears.

Temperatures should be taken rectally or vaginally with a reliable rectal type clinical thermometer, which is left in place at least 5 minutes. The temperatures must be taken at the same time every day, setting an alarm clock, if necessary, to rouse the

patient on days when she wishes to sleep later. The patient should have had at least 6 hours of restful sleep and 8 to 12 hours without food for reliable temperatures. She should note the presence of upper respiratory or other infections, or interruptions to sleep, nightmares, gastro-intestinal distress, or other circumstances which might affect the temperature on any particular day. Before the temperatures can be used for information regarding the sex cycle, a record of several months' duration should be available. On examination of the record it will be seen that the cyclic fluctuation occurs around an average value which is characteristic for each patient. The average itself fluctuates slightly seasonally.

It has been demonstrated that the correlation between the temperature and gonad

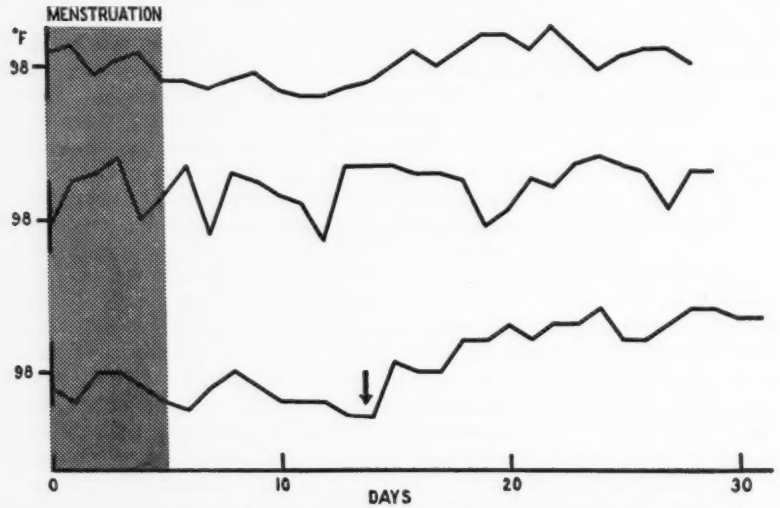

Fig. 1. Temperature curves of 3 types. The uppermost curve is the commonest variety associated with an anovulatory cycle. The range of temperatures is 0.9°F. The rate of change from day 12 to day 16 of the cycle is about 0.2°F. per day. The gradual change (in contrast with the sharp, ovulative change in the lowest curve) is indicative either of atresia or possibly luteinization of an unruptured follicle. The middle temperature curve shows a number of large changes but no rhythmic downswing followed by a sustained rise. It is common in women approaching the menopause. It may indicate failure of follicle development. The lowest curve is that of a normal ovulative cycle. Coitus (at the arrow) was followed by conception and pregnancy.

function takes the following course: during the phase of follicle development and increasing estrone production, the basal rectal temperature tends to drop progressively. The low point in the temperature curve is reached when the follicle matures, that is just before ovulation. Coitus at this time is therefore most likely to result in conception. Even the minimal progesterone production which occurs preovulatively suffices to counteract in part the temperature depressing action of estrone, and therefore, to cause an initial temperature rise beginning a few hours before ovulation. The temperature rise continues after ovulation and should exceed 0.5°F. in the first 24 hours postovulatively and should exceed 1.0°F. the first week postovulatively. Several typical temperature curves are presented in figure 1. As soon as the corpus luteum has regressed (a few days premenstrually) new follicles begin to develop. Estrone production begins again, although at a low level. Since there is now no functional corpus luteum, the temperature depressing action of estrone is again apparent. The

temperature begins to go down. It drops progressively, practically without interruption throughout the entire premenstrual phase of the cycle. (The drop continues on through the preovulative phase of the next cycle.)

In the event of pregnancy, corpus luteum function naturally persists. Consequently, the temperature rise of the postovulative phase is maintained. If the temperature curves of a number of previous cycles are available for inspection, it is sometimes possible to detect a pregnancy before the period is missed, since the temperature remains high. Certainly by the time the period is missed, the maintenance of a high temperature is a fairly reliable indicator of persistence of the corpus luteum and, therefore, of probable pregnancy. The temperature and smear technics used together have proved to be as reliable in detecting early pregnancy as the Friedman test.

In previous communications (2, 14) we have shown how the smear-temperature technic may be used in the study of normal sex cycles. Other investigators have employed this technic with advantage in controlling the treatment of menstrual disturbances (15) and in the treatment with estrogen of the climacteric syndrome (16, 17). In a recent preliminary communication (18) this technic was used in the diagnosis and treatment of sterility in women.

By use of the smear-temperature technic it has been possible to classify functional sterilities in three etiological varieties, a detailed description of which we now present.

The commonest type of functional sterility is that type in which the cycles are anovulatory. In my group of 48 cases, 23 fall into this classification. In considering anovulatory cycles, it is well to remember that patients who fail to ovulate in 3 to 6 successive cycles may ovulate immediately thereafter. This is very clearly borne out by the fact that of 23 patients with anovulatory cycles 6 did eventually become pregnant without any treatment. While in previous cycles there had been no ovulation they finally did ovulate as was shown not only by the smears and temperatures, but by the conception with coitus restricted to the day of lowest temperature and cornified vaginal smear. However, since in these patients there had been no previous conception in from 3 to 10 years of married life (no contraceptives were used) it seems fair to assume that their ovulative cycles had been at least infrequent.

The smear-temperature characteristics of the normal ovulative cycle have already been described. By contrast with a normal ovulative smear which shows marked desquamation of Types III, IV and V cells and an influx of leucocytes; the smears of the anovulatory cycle show the marked desquamation characteristic of ovulation. While the preovulative phase of the cycle often appears to be normal, and the temperatures show the expected gradual and progressive drop, there is no clear-cut and sudden temperature rise. Instead, the temperatures rise gradually in steps of approximately 0.2 of a degree. This type is commonest in young adult women. In older women, however, the normal preovulative development of the follicle appears frequently to be absent. The temperatures show no consistent curve and the smears show only minor daily fluctuations. The amenorrheic anovulatory cycle may be dismissed with a characterization that there is no cycle in the temperatures and that the smears persistently show cell Types VII, VIII and I.

Until recently, the possibility that menstrual cycles apparently normal in duration and frequency could occur without ovulation was denied. Hartman (19) summarizes and reviews critically the work on which our change of opinion is based. The opinion is still generally held that anovulatory cycles are rare except in adolescence, at the climacteric and during lactation, i.e. during periods of readjustment of the reproductive apparatus.

The present report of the frequency of anovulatory cycles is based upon a study of 739 cycles of 101 patients and subjects distributed as follows: 110 cycles of 19 normal women, ages 22 to 35 years; 184 cycles of 16 psychoneurotic women, ages 26 to 37; 445 cycles of 66[4] patients whose complaint was sterility and whose age range was 19 to 43 years. Of the 739 cycles, 338 seemed anovulatory by the criteria employed; no patient was exempt from at least a single anovulatory cycle.

It seems fair to note that the patients studied probably represent a relatively infertile group. Normal women are not readily available for such a study. Consequently it would be dangerous to draw any conclusions regarding frequency of ovulation in the general population from the above statistics. The study does suggest, however,

TABLE 1. SUMMARY OF CAUSES OF FUNCTIONAL STERILITY AS DETERMINED BY THE VAGINAL SMEAR, BASAL-BODY TEMPERATURE TECHNIC AND RESULTS OF TREATMENT

	Total no. patients	Total no. cycles	No. ovulative cycles	Pregnancies	Abortions	No results
Anovulatory cycles	25	106	8	6	3	19
Anovulatory cycles treated with gonadogen[1]	19	51	14	8	1	10
Failure of implantation	4	35	22	0	0	4
Early abortion	10	62	50	18	11	3
Ovulation during menstruation	4	11	6	4	0	0
Male sterility factor	5			1	1	5
Total	67	264	110	36	15	36
Duplicates	19	66	0	4	4	9
Net total cases	48	198	110	32	11	27

[1] Gonadogen, Upjohn, supplied through the courtesy of Dr. E. Gifford Upjohn of the Upjohn Co. Gonadogen is equine gonadotropin. Its use will be described in a future publication.

that anovulatory cycles occur much more frequently than had heretofore been recognized.

Besides anovulatory cycles, the other causes of 'functional' sterility in order of their importance are: *a*) early abortion, *b*) failure of implanation, and *c*) ovulation at times when the patients avoid coitus.

Early abortion. Ten of the 48 patients studied were definitely not sterile. They had normal temperature curves and showed the normal smear changes of the ovulative cycle. Their cycles, however, were unusually irregular often more than 40 days in duration. Since in these patients on several occasions the smear-temperature technic indicated persistence of a functional corpus luteum beyond its normal span, Friedman tests were made. These were positive for pregnancy. These pregnancies of which the patients themselves were unaware terminated with cramps, profuse flow, passage of clots and shreds and in 4 of the 10 cases with recovery of the fetal membranes. It is interesting to note that in 8 pregnancies in which the ovulation had been induced by equine gonadatropin only two terminated in early spontaneous abortion.

Failure of implantation. Sterility could reasonably be attributed to failure of implantation in only 4 of the patients. These patients had normal preovulative and ovulative phases according to both smears and temperatures. The smears, about 3 days after ovulation, showed a sudden regression, i.e. the sudden appearance of Type VII cells after an extremely short progesterone phase. The temperatures also dropped fairly suddenly, and stayed at an intermediate level until the onset of the next men-

[4] The 48 sterility patients of a preceding paragraph are patients on whom my studies have revealed the cause of sterility to my satisfaction. The additional 18 cases represent incompletely studied cases of which one or more cycles could be used.

strual flow, 7 to 10 days later. It is reasonable to infer that in these patients not enough progesterone was secreted to maintain the secretory phase of the endometrium until after implantation had occurred. This is further borne out by the fact that in 2 of these patients premenstrual endometrial biopsies had been made before they were referred to me. The endometrial biopsies had showed 'atrophic secretory endometrium.' So far no treatment has been successful in modifying their cycles sufficiently for pregnancy to occur.

Ovulation during menstruation. Four of the patients were sterility cases only because of the fact that they ovulated during the period of menstrual flow and avoided coitus at that time. In these patients, the temperatures dropped precipitously during the menstrual flow and rose sharply immediately the flow was over, to remain high until the premenstrual phase. Their smears during the menstrual flow were difficult to evaluate because of the presence of so much blood, mucus and leucocytes. Nevertheless, it was evident that cell Types III and IV were predominant. When these patients were advised to disregard their scruples and perform coitus during the period of flow (two on day 4, one on day 5 and one on day 6 of the cycle) they conceived and have carried their pregnancies to term without complications.

It is interesting to note that in many of these cases the sterility cannot be attributed to persistent gonadal hypofunction in the one partner. In 5 of the patients' husbands, there was a clear-cut oligospermia and more than 25% necrospermia. These patients represent a not unusual difficulty: the gonad function of both partners must be simultaneously improved if the sterility is to be treated successfully.

Since by the vaginal smear-basal body temperature technic it is possible to discover what gonadal insufficiency leads to the sterility, this technic points out the phase of the cycle in which therapy may be applied with greatest hopes for success. Since this method distinguishes between estrone and progesterone deficiencies, the type of therapy is also suggested.

The process of investigation of functional sterility, therefore, comprises the following stages.

The attempt to locate the time of ovulation.

The determination of any hormone deficiency in either the pre- or postovulative phase of the cycle.

The attempt to influence the cycle by means of appropriate therapy controlled by the vaginal smear-basal body temperature technic.

In the event that ovulation is found to occur, coitus is advised on the preovulative and ovulative days, regardless of on what day in the cycle ovulation may occur. If ovulation does not occur, therapy is directed to its induction. In the event that the sterility is due to a deficiency in the corpus luteum phase of the cycle, luteinizing hormone or progesterone would seem to be indicated.

SUMMARY

A procedure has been presented for evaluating vaginal smears and basal body temperatures as they relate to the sex cycle of women.

It has been pointed out that the presence of infection or chronic irritation may invalidate the interpretation of smears and temperatures.

Evidence has been presented indicating a greater than heretofore reported frequency of anovulatory cycles.

A scheme for the diagnosis and management of functional sterility in women has been presented.

I am glad to take this opportunity to acknowledge the inspiration and advice of the late Dr. T. Wingate Todd without whose aid and guidance this work would have been impossible. I am grateful to Dr. William H. Weir for making available clinical facilities in the Gynecological Out-Patient Department of Lakeside Hospital and to Doctors J. L. Reycraft and N. E. Clayman for their friendly interest in the work and their cooperation in securing suitable subjects for these studies.

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THE ACTION OF VARIOUS STEROID HORMONES ON THE OVARY

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IN COMPARISON WITH the extensive literature concerning the gonadotropic functions of the pituitary, relatively little has been written about the effects of the steroid hormones on the ovary.

Adrenal cortical hormones. Earlier investigators working with a variety of animal species obtained conflicting results concerning the actions of their preparations on the female gonad. Some (1–3) could find no effect on the development of the immature mouse ovary, while others (4–9) obtained positive effects. Similarly in the immature (10–20) and mature rat (18, 19, 21–30) the reported findings were contradictory. At the present time, however, knowing that the adrenal cortex contains a host of active steroids it appears probable that the differences in the results of the earlier investigators were due to differences in their crude extracts, or—if they used transplants—to the fact that these produced several hormones.

As regards pure cortical preparations there are only a few reports on desoxycorticosterone. Hoffmann (31–33) obtained no effect on the ovary with this substance although previously he had noted gonad stimulation following administration of crude extracts. Selye (34) found, on the other hand, that large doses of desoxycorticosterone acetate caused a moderate stunting of somatic growth and marked inhibition of gonadal development in the immature rat. He also noted that this inhibition lasts for a considerable length of time after the hormone injections are discontinued, and that in the adult rat a slight atrophy of the ovary is produced in the case of prolonged treatment with moderate doses.

Corpus luteum hormone. Beard (35) and Prenant (36) were the first to advance the theory that the corpus luteum of pregnancy is in some way responsible for the fact that no follicles mature and no corpora lutea are formed as a rule during the gestation period. However here again the experimental work of subsequent investigators yielded contradictory results (37–44). More recently, Selye et al. (45) administered 4 mg. of synthetic progesterone daily to normally cyclic adult rats and noted immediate cessation of the vaginal cycles, while autopsy on the thirteenth day of treatment revealed atrophic ovaries. On the other hand, McKeown and Zuckerman (46) claimed that daily administration of 1 mg. of progesterone during 9 to 11 days elicits the formation of new corpora lutea; however, as they used post pubertal rats there is no reason to believe that these corpora were formed as a result of their treatment rather than in spite of it. We shall see later that in our experiments, this dose was never sufficient to produce such an effect.

Testosterone. Somewhat more agreement is found in the literature regarding the action of this hormone. In the rat, ovarian atrophy was obtained by some workers (47–48) while others observed stimulation of corpus luteum formation (46, 48–49). Korenchevsky (50) has put forward a compromise view with which the above observations would be in agreement, viz., that short treatment with testosterone increases the size of the ovaries eliciting the formation of numerous corpora lutea, while prolonged treatment results in ovarian atrophy. More recently, Selye (68) produced follicular cysts with testosterone in mice.

Estrin. In the mouse, it has been fairly well established that large doses given over a short period of time lead to corpus luteum formation (51–53), while continuation of the same high dose, or chronic low dose treatment result in follicular atresia and corpus luteum involution (54–55). Similarly in the rat if small doses (2 to 4 U) are given daily, ovarian

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atrophy ensues, although after a time adaptation seems to occur with the restoration of normal appearance and function of the ovary (56–57). On the other hand, the ovaries of mature rats respond to large doses of estrin given for a short time (about 2 weeks) with the formation of large corpora lutea similar in appearance to those seen during gestation (58–62). Continued administration of the large doses cannot maintain these corpora which involute after a few weeks (60).

Combined administration of several steroids. Selye and Stevenson (63) noted in the mouse that the ovary became atrophic when the animal was treated with estradiol alone, while no atrophy was seen if progesterone was simultaneously administered. Selye (64–65) confirmed this for the rat and found also that testosterone propionate could likewise prevent the gonadal atrophy caused by estradiol. On the other hand, he noted that testosterone propionate failed to prevent the ovarian atrophy caused by progesterone.

In view of the foregoing experiments, it appeared of interest to investigate the gonadotropic effects of desoxycorticosterone acetate about which little is known, and of progesterone, about which there is controversy. We also wished to extend

TABLE 1

Treatment	No. animals	Body wt.	Duration	Ovary wt.	P	Histology
		gm.	*days*	*mg.*		
Cholesterol	4	18 (16–20)	10	3.5 (3.0–4.0)		Corpora lutea present. Normal ovarian structure.
Cholesterol	5	17 (16–18)	20	3.9 (3.7–4.0)		
Desoxycorticosterone acetate	4	19 (18–21)	10	4.0 (3.0–5.0)	0.3	No corpora lutea in any ovary. Very many follicles undergoing cystic atresia. Stroma atrophic.
Desoxycorticosterone acetate	4	18 (16–19)	20	3.1 (3.0–3.2)	<0.01	

the observations of Selye et al. on the effects of combined administration of steroid hormones with the view of finding an explanation for the apparently paradoxical effects which they noted.

Desoxycorticosterone Acetate

Experiment I. Female, non-pregnant, adult mice of the strain designated as DBA (Little's dilute brown strain) were used. Four animals received daily subcutaneous injections of 3 mg. of desoxycorticosterone acetate dissolved in 0.2 cc. of pure peanut oil, while 4 controls were treated with the same amount of cholesterol in oil. A similar second series was used for a more chronic experiment. The significance of the apparent differences between the treated and control groups was evaluated by "Student's" method for small samples and is expressed in the table in terms of probability, estimated by graphic interpolation in Fisher's table of t. In accordance with the usual convention, differences between series' cannot be accepted as significant when P is greater than 0.05. In calculating the significance of the apparent changes, the 10-day cholesterol treated animals served as controls for the 10-day desoxycorticosterone acetate treated animals, and similarly the 20-day desoxycorticosterone acetate group was compared with the 20-day controls. The results of both experiments are summarized in table 1.

From this experiment, we conclude that brief treatment results in complete absence of corpora lutea, increased number of follicles undergoing cystic atresia, and stromal atrophy. It is accompanied by a slight (non-significant) increase in

weight. Prolonged treatment accentuates these changes, and is reflected in a significant loss of gross weight (fig. 1, 2).

Experiment II. Six newborn female hooded litter-mate rats were selected. Of these 5 animals received daily from the day of birth 1 mg. of desoxycorticosterone acetate in 0.2 cc. of peanut oil, subcutaneously for 30 days, followed by 2 mg. as above for a further 10 days. Two of the treated animals were then killed while 3 were maintained for 30 days without treatment and then killed. In all cases the ovaries were taken for weight determination and histological study.

The 2 animals killed on the 40th day of treatment, having received a total of 50

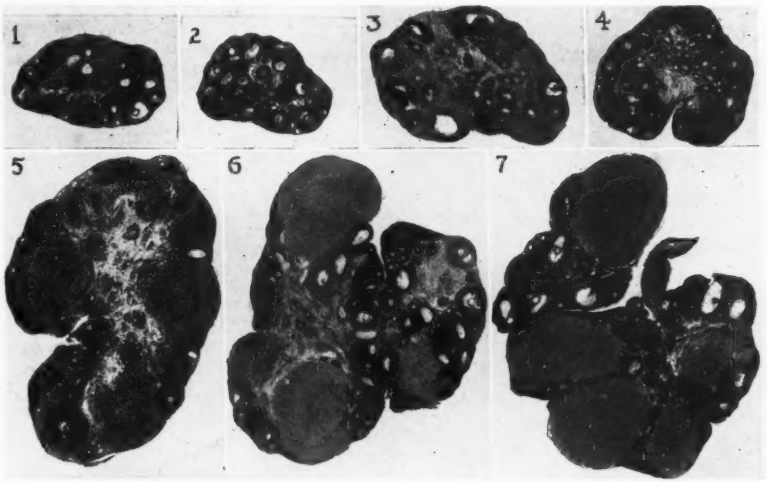

Fig. 1. ADULT MOUSE TREATED WITH 3 MG. OF CHOLESTEROL FOR 10 DAYS. Section shows normal ovarian structure. Fig. 2. ADULT MOUSE TREATED WITH 3 MG. OF DESOXYCORTICOSTERONE ACETATE FOR 10 DAYS. Corpora lutea are absent. There is an increase in the number of follicles showing cystic atresia and the stroma is atrophic. Fig. 3. FORTY-DAY-OLD HOODED RAT NOT TREATED. Section shows normal ovarian structure. Fig. 4. FORTY-DAY-OLD HOODED RAT TREATED WITH A TOTAL OF 50 MG. DESOXYCORTICOSTERONE ACETATE FROM DAY OF BIRTH. Ovary is very small and shows absence of corpora lutea, increase in number of cystic atretic follicles and very atrophic stroma. Fig. 5. ADULT ALBINO RAT, UNTREATED, SHOWING NORMAL OVARIAN STRUCTURE. Fig. 6. ADULT ALBINO RAT, TREATED WITH 10 MG. DESOXYCORTICOSTERONE ACETATE DAILY FOR 20 DAYS. Note the increased number of follicles undergoing cystic atresia, the large corpora lutea and the atrophic stroma. Such large corpora lutea were found only in half of our rats thus treated (compare with fig. 5). Fig. 7. ADULT ALBINO RAT TREATED WITH 10 MG. PROGESTERONE DAILY FOR 20 DAYS. Note the very large corpora lutea occupying most of the ovary, increase in number of follicles undergoing cystic atresia, and atrophic stroma (compare with fig. 5).

mg. of the hormone, showed ovarian weights of 6.3 mg. and 7.2 mg. or an average of 6.7 mg. as against an untreated litter mate killed on the same day which had an ovarian weight of 23 mg. The 3 animals in which treatment was suspended during the next 30 days showed at the end of that time an average ovarian weight of 9.3 mg. (6.0–7.0–15.0 mg.) which is still markedly below the normal ovarian weight of a 70-day-old rat and even markedly below the 23 mg. weight of the 40-day-old litter mate in this series. In all the hormone treated animals, the ovarian stroma was found to be markedly atrophic. No corpora lutea were present in any of the treated animals (fig. 3, 4).

Despite the fact that the data is derived from a small number of observations the weights and histology are striking so that we conclude that desoxycorticosterone

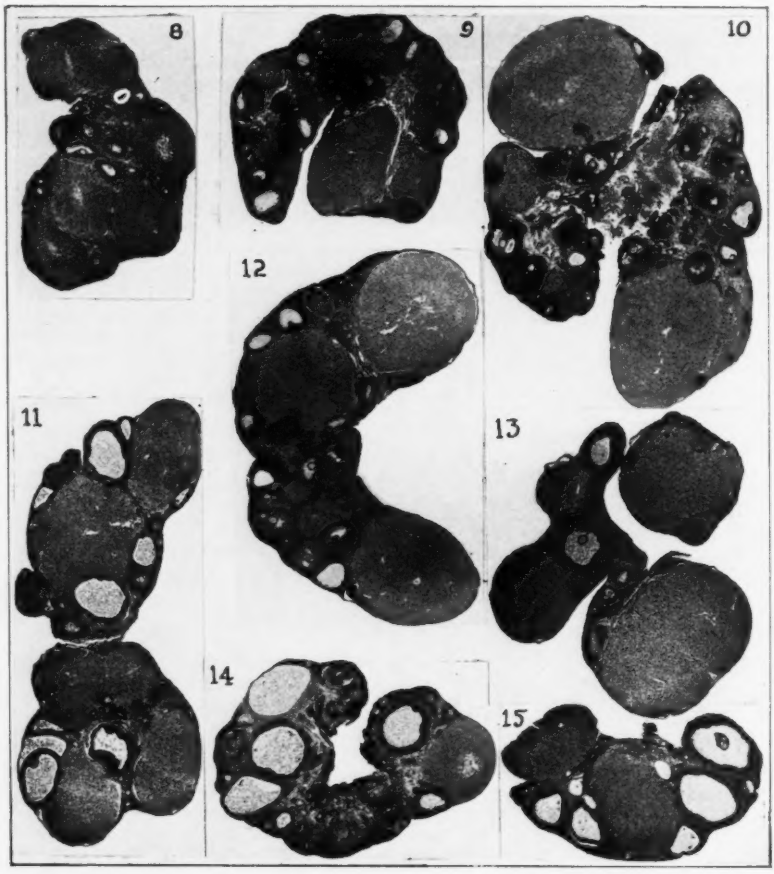

Fig. 8. ADULT ALBINO RAT UNTREATED SHOWING NORMAL OVARIAN STRUCTURE. Fig. 9. ADULT ALBINO RAT TREATED WITH 2 MG. PROGESTERONE DAILY FOR 21 DAYS. The ovary is very small and filled with many follicles showing cystic atresia. The stroma is very atrophic (compare with fig. 8). Fig. 10. ADULT ALBINO RAT TREATED WITH 300γ OF ESTRIN PLUS 2 MG. OF DESOXYCORTICOSTERONE ACETATE DAILY FOR 21 DAYS. Corpora lutea are very prominent and there is an increase in the number of follicles undergoing cystic atresia. Stroma is very atrophic (compare with fig. 8). Fig. 11. ADULT ALBINO RAT TREATED WITH 300γ OF ESTRIN PLUS 2 MG. OF TESTOSTERONE DAILY FOR 21 DAYS. The corpora lutea are conspicuous as are the beginning follicular cysts. The stroma is atrophic (compare with fig. 8). Fig. 12. ADULT ALBINO RAT TREATED WITH 300γ OF ESTRIN PLUS 2 MG. OF PROGESTERONE DAILY FOR 21 DAYS. Note conspicuous corpora lutea, numerous atretic follicles and atrophic stroma (compare with fig. 8). Fig. 13. ADULT ALBINO RAT TREATED WITH 300γ OF ESTRIN PLUS 2 MG. OF TESTOSTERONE AND 2 MG. OF PROGESTERONE DAILY FOR 21 DAYS. Very large corpora lutea occupy most of the section, 2 beginning follicular cysts are conspicuous. Stroma is very atrophic (compare with fig. 8). Fig. 14. ADULT ALBINO RAT TREATED WITH 2 MG. OF DESOXYCORTICOSTERONE ACETATE PLUS 2 MG. OF TESTOSTERONE DAILY FOR 21 DAYS. Ovary is very small and contains 4 prominent follicular cysts and numerous atretic follicles. The stroma is atrophic (compare with fig. 8). Fig. 15. ADULT ALBINO RAT TREATED WITH 2 MG. OF TESTOSTERONE PLUS 2 MG. OF PROGESTERONE DAILY FOR 21 DAYS. Note the large corpora lutea and prominent beginning follicular cysts. Stroma is atrophic (compare with fig. 8).

acetate inhibits the development of the immature rat ovary producing marked stroma atrophy and preventing the formation of corpora lutea. This effect is still operative for a long time after cessation of the injections.

Experiment III. Six newborn female litter-mate albino rats of an average body weight of 17 gm. at the beginning of the experiment (8th day of life) were selected. Three of the animals received 1 mg. of desoxycorticosterone acetate in 0.2 cc. subcutaneously every 2 days, while the remaining 3 served as untreated controls. One animal in each group was killed on the 30th and the remaining 2 in each group on the 37th day of life. The ovaries in all cases were taken for weight determination and histological study.

On the 30th day of life the ovaries of the treated animal weighed 8.0 mg. (body weight 68 gm.) as against an ovarian weight of 12 mg. (body weight 65 gm.) in the untreated control killed on the same day. On the 37th day of life the treated animals had an average ovarian weight of 9.7 mg. (7.4–12.0 mg.) as against 19 mg. (11.0–27.0 mg.) in the untreated animals killed on that day. All 4 animals had body weights of 80 to 87 gm.

This experiment furnishes further evidence that desoxycorticosterone acetate inhibits the development of the immature rat ovary.

Experiment IV. Two groups of 12 adult female albino rats were used. In the first group, 6 animals received daily 2 mg. of desoxycorticosterone acetate subcutaneously and 6 served as untreated controls. In the second group, 6 received 10 mg. of desoxycorticosterone acetate in 0.4 cc. of peanut oil daily subcutaneously while the remaining 6 served as untreated controls. At the end of the period of injection all the animals were killed and the ovaries taken for weight determination and histological study. Table 2 summarizes the data.

TABLE 2

Treatment	No. animals	Body wt.	Ovary wt.	Histology
		gm.	mg.	
Desoxycorticosterone acetate 2 mg.—21 days	6	135	33.0	Marked increase in number of follicles undergoing cystic atresia. Stroma very atrophic.
Untreated	6	142	36.0	Normal ovarian structure.
Desoxycorticosterone acetate 10 mg.—20 days	5	142	26.0	Same as in above desoxycorticosterone acetate but some enlarged corpora.
Untreated	6	148	37.0	Normal ovarian structure.

We see that in the adult rat prolonged treatment with relatively small doses of desoxycorticosterone acetate elicits follicle changes with stroma atrophy and is accompanied by an insignificant loss in ovarian weight. In 2 animals of this group the corpora lutea were large so that despite the stroma atrophy, in these the ovarian weights were 52 and 53 mg. respectively. If these were eliminated the loss of weight from the stroma atrophy would be still more apparent.

Prolonged treatment with a high dose of desoxycorticosterone acetate produces the same cystic degeneration of numerous follicles giving the ovary a "Swiss cheese" appearance, and the same stroma atrophy as above. However in this case there is a pronounced loss of ovarian weight. Both in high and low dose treatment cystic atresia of follicles is often seen. In the rat, desoxycorticosterone acetate usually causes no corpus luteum formation in the doses employed here, although definitely large corpora have been noted in some cases (fig. 5, 6).[1]

[1] As an incidental observation we noted that in all cases treated with desoxycorticosterone acetate there was an unusually large amount of free peritoneal fluid. An analysis of the mechanism responsible for this phenomenon will be published later.

Corpus Luteum Hormone

Experiment I. Twelve adult female albino rats having an average body weight of 151 gm. were divided into 2 groups. Six received 21 daily subcutaneous injections of 2 mg. of progesterone in 0.2 cc. of peanut oil, while the remaining 6 served as untreated controls. At the end of the injection period, the animals were killed and the ovaries taken for weight and histological study.

In the treated group, the ovaries weighed only 22.9 mg. (11.2–31.6) as compared to 36 mg. (33.5–40.1) in the normal controls. The ovaries of the treated group were noteworthy in that although degenerating corpora lutea were present in about the same proportion and size as in the controls, completion of follicle maturation was markedly depressed because many of them had undergone cystic atresia. The stroma was very atrophic showing beginning 'wheel cell' formation.

We conclude that in the rat prolonged treatment with low doses of progesterone depresses follicle maturation and causes stroma atrophy, but apparently exercises no influence on the corpora lutea. The atrophy is reflected in a significant loss of ovarian weight. ($P = <0.01$).

Experiment II. Five adult female albino rats having an average body weight of 154 gm. received 20 daily subcutaneous injections of 10 mg. of progesterone in 0.4 cc. of peanut oil, while 6 animals of the same weight served as untreated controls. At the conclusion of the period of injection the animals were killed and the ovaries taken for weight determination and histological study. In the treated group the average ovarian weight was 40 mg. (20–50) as compared to 37 mg. (33–43) in the controls. If we omit one animal whose ovaries were abnormally small (20 mg.) the increase in ovarian weight in the treated group is even more significant, as the other 4 had ovaries ranging between 30 and 50 mg. In our rat colony, spontaneous ovarian atrophy is occasionally seen although its cause is not definitely known. This may explain the one instance cited above. In the other ovaries, large corpora lutea accounted for the rise in weight, although these were involuting at the time of autopsy. However, the marked follicular atresia and stroma atrophy, in many cases accompanied by beginning wheel cell formation, were striking (fig. 5, 7).

In the adult rat, a marked depression of follicle maturation and stroma atrophy are seen after treatment with high doses of progesterone. However, since such treatment also causes ovulation and the formation of large corpora lutea, the gross weight of the gonad may be above normal in spite of this.

Combination treatments. In the series of experiments tabulated below, all animals used were adult female albino rats having an average body weight of 134 gm. They were injected subcutaneously with various steroids, the daily dose of which was dissolved in 0.2 cc. of peanut oil in each case. After 21 injections all animals were killed and the ovaries were weighed and taken for histological study. Table 3 summarizes our results. Each ovarian weight and histological description represents the average of a group of 6 identically treated rats. From these data it appears that in 2 mg. doses given 21 days, desoxycorticosterone acetate, testosterone or progesterone do not cause the formation of large, 'pregnancy-type' corpora lutea, but lead to marked involution of the stroma and consequently to a decrease in ovarian weight. Daily administration of 300γ of estradiol for the same period likewise results in atrophy of the stroma and a decrease in the gross weight of the ovary. However, histological examination of the latter series still shows remnants of fairly large corpora lutea. Direct inspection after 14 days of treatment in this series showed no large corpora lutea in the groups treated with desoxycorticosterone acetate, testosterone or progesterone but revealed the presence of hyperemic, large 'pregnancy-type'

corpora in all groups in which estradiol was given alone or in combination with other hormones. The fact that at autopsy on the 21st day of treatment the ovarian weights were below normal in the groups receiving estrin alone or in combination with desoxycorticosterone acetate is attributed to the circumstance that this dose of estradiol either by itself or even in combination with desoxycorticosterone acetate was unable to maintain the large corpora lutea which it originally produced. Similarly in the groups receiving desoxycorticosterone acetate in combination with testosterone or progesterone, the ovaries were small because the stroma underwent

TABLE 3

Treatment	Ovary weights	Histology
	mg.	
Untreated	36.0	Normal ovary
Estradiol 300γ	30.0	Arrophic stroma—corpora lutea of moderate size; involuting.
Desoxycorticosterone acetate 2 mg.	33.0	Atrophic stroma—increase in number of follicles undergoing cystic atresia.
Testosterone 2 mg.	25.0	Atrophic stroma—follicles in all stages with beginning cyst formation.
Progesterone 2 mg.	22.9	Atrophic stroma—scarcity of maturing follicles.
Estradiol 300γ Desoxycorticosterone acetate 2 mg.	32.0	Atrophic stroma—corpora lutea of moderate size; involuting.
Estradiol 300γ Testosterone 2 mg.	40.0	Atrophic stroma—large corpora lutea—small follicular cysts.
Estradiol 300γ Progesterone 2 mg.	46.0	Atrophic stroma—large corpora lutea—relative scarcity of follicles.
Estradiol 300γ Progesterone 2 mg. Testosterone 2 mg.	41.0	Atrophic stroma—large corpora lutea—relative scarcity of maturing follicles—beginning follicular cysts.
Desoxycorticosterone acetate 2 mg. γ Testosterone 2 mg.	22.0	Atrophic stroma—small follicular cysts.
Progesterone 2 mg. Testosterone 2 mg.	26.1	Atrophic stroma—scarcity of maturing follicles—some follicle cysts.

atrophy and no 'pregnancy-type' corpora were formed. On the other hand, in the groups treated with estradiol simultaneously with progesterone, testosterone or both of these latter hormones, the ovarian weight was above normal because in these combinations, this dose of estradiol maintained the large corpora lutea for 21 days. The stroma atrophy produced by all the hormones used in this series was in no way inhibited in any of the groups given combinations of sterols (fig. 8–15). These observations clarify the apparently paradoxical finding that the ovaries may actually become larger than normal in case of combined treatment with two hormones each of which decreases ovarian weight. The fact that progesterone and testosterone given simultaneously each in 2 mg. doses cause no large corpora or increase in ovarian weight is probably due to the circumstance that even 4 mg. of either of these hormones is not sufficient to produce 'pregnancy corpora.' From other experiments, we know however, that in 8 to 10 mg. daily doses these steroids cause the formation of such corpora, so that this is probably merely a matter of dosage.

The question whether the gonadotropic effects of the above steroids are due to their direct action on the ovaries or are mediated through the pituitary is also of importance. That steroids can exert a direct gonadotropic effect was first shown by Selye and Collip (66). They found that in hypophysectomized rats, the ovaries become atrophic and cannot be stimulated by estrone, but if this atrophy is prevented by maintenance doses of pituitary gonadotropic substances, estrone has a direct effect on the ovaries inasmuch as it produces 'pregnancy-type' corpora. Recently Pencharz (67) and others have come to similar conclusions.

It is difficult to explain the fact that the various steroid hormones may either stimulate or inhibit the ovary, yet this dual action has also been noted with respect to their effect on the testis (64, 65). In the latter case, the situation is somewhat less complicated because the confusing factor of qualitatively different actions (stroma atrophy and corpus luteum formation) is absent. All our observations may perhaps best be explained by assuming that the gonad inhibitory action of the steroids is indirect and due to the depressing effect which they exert on the gonadotropic hormone production of the hypophysis, while their gonad stimulating action is direct. In good accord with this interpretation, we found that none of the steroid hormones cause atrophy of the gonads in hypophysectomized animals receiving maintenance doses of hypophyseal gonadotropic preparations. It seems probable that estrin for instance, which stimulates the corpora lutea in hypophysectomized rabbits even without gonadotropic hormone treatment, while in the rat simultaneous administration of the latter is essential to secure this effect, acts in this manner because the involution of the ovary is more rapid in the rat and consequently the gonad loses its responsiveness unless it is maintained by a pituitary preparation. The male gonads on the other hand, which involute less rapidly in the hypophysectomized rat, are readily stimulated by such steroids as the various androgens and progesterone. This will receive more detailed consideration in a future publication dealing with the effect of steroid hormones in the male. It also appears that, at least in the female, smaller doses of the various steroids suffice to exert the indirect gonad inhibitory action than are required to demonstrate the direct gonad-stimulating effect. This was true in the case of estrogens, progesterone and androgens. Desoxycorticosterone acetate proved inhibitory in most cases except in some of the rats receiving 10 mg. daily so that essentially its action is the same as that of the other steroids. This interpretation might perhaps help to understand why, despite the great differences in their specific effects, the actions of the various steroid hormones on the gonad are so surprisingly similar.

SUMMARY

Desoxycorticosterone acetate inhibits the development of the immature rat ovary, while in the adult rat and mouse it produces atrophy of the stroma and cystic atresia of follicles. Only in some animals receiving 10 mg. daily did it elicit the formation of enlarged corpora.

Progesterone given in daily doses of 2 mg. causes only atrophy of the stroma and inhibition of follicle maturation in the rat. In doses of 10 mg. per day it leads to the formation of a single set of 'pregnancy-type' corpora lutea, still inhibiting follicle maturation and causing stroma atrophy.

Testosterone elicits only stroma atrophy and the formation of follicle cysts when given in doses of 2 mg. per day. However other experiments performed in this laboratory but not reported here in detail indicate that if 8 daily doses of 6 mg. are administered to the rat, testosterone also leads to the formation of a single set of large corpora lutea. More prolonged treatment even with such large doses results in the gradual involution of these corpora and the formation of follicle cysts.

Estradiol in daily doses of 300γ produces only very short-lived 'pregnancy-type' corpora. However, if this amount of estradiol is given in combination with testosterone or progesterone, the life span of the newly formed corpora lutea is greatly prolonged although the dose in which these latter hormones are administered is too small to cause corpus luteum formation by itself. This explains the surprising fact that the ovarian weight increases if the dose of estrin which decreases ovarian weight by itself is administered simultaneously with a dose of testosterone or progesterone which given alone would also cause only ovarian atrophy. The maintenance of the large corpora lutea in such cases overcompensates for the relatively small loss in ovarian weight which results from the stroma atrophy.

It is noteworthy that testosterone retains its follicle cyst forming effect when given in combination with estradiol, progesterone or both these hormones. The ability of estradiol, progesterone and testosterone to form and maintain large 'pregnancy-type' corpora lutea as well as their power to cause atrophy of the ovarian stroma, are summated in case of combined treatment with such hormones, while the follicle cyst-forming effect of testosterone is not significantly influenced by other steroids.

The view is expressed that the various steroid hormones exert a gonad-inhibitory action which is indirect and due to the depression of the hypophyseal gonadotropic hormone production. This common pathway in the mechanism of their action may explain why diverse steroids produce the same type of atrophy. The gonad stimulating effect characteristic of most, if not all, the above mentioned steroids on the other hand, appears to be direct as it is not prevented by hypophysectomy.

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THE ESTRIN CONTENT OF THE FOLLICULAR FLUID AND URINE OF THE MARE AND ITS RELATION TO PHENOMENA OF THE ESTRUAL CYCLE[1]

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ALTHOUGH the literature is replete with studies of the concentrations and interrelations of the hormones during the physiological state of pregnancy in the mare, investigations involving similar studies during the various phases of the estrous cycle of the non-pregnant mare are rare. Zondek (1) reported comparative data upon the estrogenic hormones in the urine of the pregnant and non-pregnant mare, and more recently Cole and Goss (2) cited the presence of gonadotropic hormone in the blood of the latter. However, nowhere in the literature are studies available upon the estrogens of the urine or follicular fluid in relation to the various phenomena of the estrual cycle.

For periods beginning in February or April and ending about the middle of July of the years 1937, 1938, and 1939 detailed studies were made in the physiology of the non-pregnant mare at the United States Range Live-Stock Experiment Station, Miles City, Montana. Forty-two purebred and grade Belgian, 2 grade Percheron, 1 grade Shire and 35 grade Thoroughbred mares were observed. Specimens of urine and follicular fluid were also obtained and sent to Columbia, Missouri for analysis and assay. The results of the assays and analyses were correlated with the physiological observations in an effort to discover any existing relations between hormone concentration and the phenomena of estrus.

EXPERIMENTAL

Follicular fluid. As a preliminary step in the collection of the follicular fluid, the ovaries of the maries under observation were palpated per rectum at different stages of the cycle, then desirable follicles were punctured and their contents collected by a method recently described by Andrews and McKenzie (3).

Prior to ascertaining the hormone content of the fluid, the volume, PH, specific gravity, and total nitrogen were determined by the usual accepted methods.

The determination of the estrogens was made by a chemical procedure in which a photoelectric colorimeter was used and the results expressed in terms of gamma of crystalline estrone.

Samples varying from 0.2 cc. to 5.0 cc. were taken for the chemical assay of the estrogens depending upon the amount of follicular fluid available. In all cases the volume was adjusted to 5.0 cc. by the addition of distilled water after which enough concentrated HCl was added to lower the PH to 3. The acid fluid was then heated for 10 minutes in a water bath of 80° to 90°C. This procedure resulted in the complete

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precipitation of all proteins which were removed by filtration. The estrogens were extracted from the protein-free filtrate by successive portions of peroxide-free ether. The ether extracts were combined, washed with 10% sodium carbonate and with distilled water, then evaporated. The formation of the color was accomplished by the addition of a phenolsulphonic acid reagent prepared by making several modifications in the method described by Cohen and Marrion (4) then following the procedure as recommended by them for the subsequent steps leading to the formation of the colored estrogen compound with the reagent. Before making the colorimetric determinations the colored fluid was cooled to 20°C. and filtered through ashless paper to remove a waxy contaminant which influenced the accuracy of the colorimetric results.

Urine samples. Since the mares under observation were range animals, the collection of 24-hour urine specimens was impracticable. These mares were brought in from the range for observation and the collection of the urine samples by catheter at the same period each day. Samples for the analyses were selected on the basis of their specific gravity in order to assure uniformity in their concentration. Under the existing standardized conditions, the urine should be an acceptable indicator of changes in the excretion of one of its variable constituents.

Samples of 50 cc. to 100 cc. were used for the estrogen assays. Hydrolysis of the urine was performed by the method recommended by Smith and Smith (5). Most of the brown-colored contaminants could be removed by adding 5 gm. of sodium bisulfite to the hydrolyzed sample and chilling in an ice-salt bath for 30 minutes. After filtering the urine was extracted with peroxide-free ether, the ether extract freed from most of the residual urinary pigments by washing with successive 20 cc. portions of 20% sodium carbonate followed by a final distilled water wash, then evaporated. The residue was dissolved in 25 cc. of toluene and the estrogens removed by extraction with 1 N NaOH. This concentration of sodium hydroxide removes both estrone and estriol, according to Cohen and Marrion (4) as well as any other estrogens whose dissociation constants lie between those of estrone and estriol. The sodium hydrozide was acidified and extracted by ether; the ether extract washed as previously described and evaporated. The estrogen concentration was determined by the photoelectric colorimeter after forming the red estrogen-phenolsulfonic acid compound and the results expressed as gamma of crystalline estrone.

RESULTS

Follicular fluid. A total of 16 follicular fluid samples were obtained from 11 mares. As indicated in tables 1 and 2, a wide variation existed in the diameter of the follicles palpated and in the total volume of fluid withdrawn from these follicles at different periods in the cycle. The diameter of the follicles ranged from 1.0 cm. to 6.0 cm. while the volumes of the follicular fluid varied from 1.0 cc. to 112 cc. In all the mares from which fluid was collected, with one exception, the size and contents of the follicles were greater during the estrous period than during non-estrus.

The one exception was *Inga* as shown in table 2. This mare was observed on the third day of estrus and a corpus luteum detected on the right ovary. Receptivity continued up to the seventh day of estrus. On the sixth day a 3.5 cm. follicle was detected and estrus was terminated the following day although the follicle failed to rupture. By the sixth day following the termination of estrus when this follicle was ruptured it had reached a diameter of 6.0 cm. and contained 112 cc. of fluid.

In the mares with normal cycles the concentration of the ether-soluble estrogens varied directly with changes in the volume of the fluid or the size of the follicles

TABLE 1. ESTROGEN CONTENT OF FOLLICULAR FLUID

Mare	Size of follicle	Volume fluid recovered	Total estrone in fluid recovered	Estrone per 100 cc. fluid	Sex behavior at time of rupture
	cm.	cc.[1]	γ[2]	γ[2]	
Idlewild	5.5	100.0	2500.0	2500	Estrus
Islet	4.0	72.0	1800.0	2500	Estrus
Helen	3.5	71.0	1863.8	2625	Estrus
Folly	2.0	35.0	735.0	2100	Non-estrus
Jenny	2.5	35.0	717.5	2050	Non-estrus
Flora I 3-4-39	2.0	3.7	59.0	1600	R.O.
Flora II 3-4-39	1.0	1.0	26.0	2625	R.O.
Flora III 3-4-39	1.5	1.2	21.9	1826	L.O.
Lottie I 3-14-39	1.3	3 small follicles	35.8	2750	
Lottie II 3-14-39	1.2	2 small follicles	22.5	1875	

[1] Size of samples taken for analyses varied from 0.2 cc. to 5.0 cc.
[2] Total estrogens expressed in terms of γ of estrone.

therefore the least quantity of total estrogen was found in the follicles in the early stages of growth and the greatest quantity at the height of follicular growth.

However, the total estrogen concentration per unit volume of follicular fluid is approximately identical for all the samples except those of the mares designated as *U. S.* I and *U. S.* II in table 2, who in addition to showing no signs of estrus during a long period of observation showed other signs of abnormality. Even though samples of follicular fluid varying in size from 0.2 cc. to 5 cc. were assayed, the concentration of estrogens per unit volume as shown in tables 1, 2 and 3 was, in every case, nearly identical.

TABLE 2. ESTROGEN CONTENT OF FOLLICULAR FLUID, MARES WITH ABNORMAL CYCLES

Mare	Size of follicle	Volume fluid recovered	Total estrone in fluid recovered	Estrone per 100 cc. fluid	Sex behavior at time of rupture
	cm.	cc.	γ	γ	
Inga	6.0	112	2856	2500	Non-estrus
Trixie L.O. 6-22-38	3.0	38	760	2000	Irregular
Trixie R.O. 6-22-38	3.0	31	852	2750	
U.S.I L.O. 3-24-39	—	25	180	720	Non-estrus
U.S.I R.O. 3-27-39	—	50	335	670	Non-estrus
U.S.II	—	30	216	720	Non-estrus
U.S.II	—	33	234	780	Non-estrus

TABLE 3. CHARACTERISTICS OF FOLLICULAR FLUID

Mare	Estimated size of follicle	Volume fluid recovered	pH	Density at 20°C.	Proteins	Estrone
	cm.	*cc.*		*gm./cc.*	*%*	*γ/cc.*
Idlewild	5.5	100	8.05	1.025	0.51	25.0
Islet	4.0	72	7.80	1.011	0.56	25.0
Helen	3.5	71	7.80	1.008	0.61	26.0
Folly	2.0	35	8.10	1.015	0.50	21.0
Jenny	2.5	35	7.80	1.003	0.66	20.5
Inga	6.0	112	7.65	1.020	0.59	25.5
Trixie R.O.	3.0	31	7.60	1.009	—	27.5
Trixie L.O.	3.0	38	7.60	1.012	—	20.0

Color—straw; consistency—slightly sticky; taste—salty; ova recovered—none.

A summary of the characteristics of follicular fluid as determined by the various analyses is given in table 3. Although a variation of hydrogen ion concentration from a pH of 7.60 to 8.1 was found, no correlation between the pH and any of the other characteristics of the fluid nor between the pH and physiological phenomena could be found.

The protein content of the follicular fluid is of interest because it is especially low and because we found it to be, within the limits of experimental error, nearly identical for all samples of follicular fluid analyzed.

TABLE 4. URINARY ESTROGENS

Mare	Sample No.	Total estrogens as gamma of estrone per liter	Day of estrous cycle
Dolly	16	3800	1 day in
Dolly	21	9200	3 days in
Dolly	51	4760	5 days out
Dolly	70	3360	10 days out
Dolly (Pregnant)	88	8160	15 days out
Floto	36	6520	15 days out
Floto	59	8820	20 days out
Floto	85	8820	26 days out
Floto	96	17,100	26 days out
Floto (Pregnant)	126	17,100	40 days out
Flora	63	5520	3 days in
Flora	72	5600	5 days in
Flora	86	4280	5 days out
Flora (Not bred)	103	6400	10 days out
Jolly	5	1180	1 day in
Jolly	15	1140	2 days out
Jolly	34	3320	8 days out
Jolly (Not bred)	73	7000	16 days out
Folly	45	3640	2 days in
Folly	48	2880	1 day out
Folly	66	4600	5 days out
Folly (Non-pregnant)	99	6360	15 days out

Urinary analyses. In any consideration of the results of the urinary analyses, we are forced to separate the samples assayed into two distinct groups, namely, those from animals which were never bred or in which conception did not take place and those from animals which according to later tests were diagnosed as pregnant during the period of the urine collection.

In the first group which includes all of the non-pregnant mares the results of the urinary analyses (table 4) indicate definite trends in estrogen excretion. A decided rise occurring during the period of estrus is evident. This rise, as shown by the data, is followed by an early post-estrous decrease in urinary estrogens of several day's duration. The evidence for a second rise in the urinary excretion of estrogens in the non-pregnant mare is most convincing. In all the mares studied the estrogen content of their urine increased to a maximum between the tenth and fifteenth day post-estrus.

The mares in the early stages of pregnancy were similar to the non-pregnant mares in their excretion of estrogens during the estrous period in which conception occurred. They also showed a decrease in estrogen excretion immediately after estrus followed by a gradual increase as the post-estrous period advanced. In the pregnant mare, however, we found a marked rise in the urinary estrogen concentration beginning about the twenty-sixth to the thirtieth day after conception had occurred. No evidence exists to indicate that the occurrence of this decided change in estrogen excretion may not vary in individual animals. At this time also there is a change in the color produced by the reaction between the phenol-sulfonic acid reagent and the estrogens present in the urine. The color produced is a purplish or cherry red identical to that produced by the estrogens present in the urine from mid- and late-pregnancy mares.

DISCUSSION

No investigator has previously cited evidence for nor stated that the estrogen content of follicular fluid per unit volume remains constant during all phases of the estrous cycle in the mare. However, Dingemanse and Muhlboch (6) in a paper upon the concentrations of the free and bound estrogens in follicular fluid of the mare presented data which indicates that identical concentrations of estrogen are found in different samples of the fluid. These investigators used large quantities of fluid drawn by syringe from excised ovarian tissue. In 3 experiments, in which 160 cc., 2.5 l. and 0.5 l. were extracted with benzene without previous hydrolysis, they obtained 25,200 I.U., 27,000 I.U. and 25,000 I.U. per liter respectively. These results would also indicate that the concentration of estrogens per unit volume of fluid was a constant, especially when one considers the marked differences in the volumes of fluid analyzed and the possibility of wide differences in the samples secured from the ovaries of unknown slaughtered animals.

The data might possibly indicate that the concentration of hormones in the fluid of the follicles is governed by the establishment of an equilibrium between the quantity of hormone retained within the fluid and that made available to the follicles. In abnormal cases apparently the equilibrium point is not reached because other factors influence the availability of the hormones to the developing follicle. This observation should be of particular value to those who contemplate using follicular fluid as an indicator of hormone production or secretion.

The density of follicular fluid is in all cases lower than that of either blood plasma or blood serum and approaches that of cerebrospinal fluid. The analytical results also indicate that no pronounced changes in density occur during the cycle and that the slight changes which take place bear no relation to the estrual cycle.

Neither can we find evidence in the data presented which would point to the proteins of follicular fluid as a factor in the slight variations in density of the fluid or as a factor in estrual physiology.

The protein content of the fluid is about one-tenth that of blood, about the same as that of some ascitic fluids and lower than that of all other body fluids except cerebrospinal fluid and intraocular fluid. The concentration of the fluid in respect to proteins might by analogy indicate a possible mechanism for the production of the ovarian fluid.

Several investigators have established the existence of two definite points of maximum excretion of the estrogens during the human menstrual cycle, but no one has reported similar excretion peaks during estrus in other animals. The evidence for two such peaks of estrogen excretion in the non-pregnant mare is quite definite in the examples we have presented as well as in the other mares studied.

SUMMARY

The ovaries of 11 mares were palpated per rectum at different stages of the estrous cycle, then desirable follicles were punctured and their contents collected for subsequent chemical analyses and chemical assay of the estrogen concentration.

The volume of the fluid obtained ranged from 1 cc. to 112 cc., varying with the age, diameter of the follicles punctured and phase of the cycle in which they were punctured. With one exception the greatest quantity of fluid was obtained during estrus.

The protein content of the fluid averaged 0.57% and was approximately the same for all samples analyzed. The specific gravity varied between 1.008 and 1.025, but in most cases was nearer the lower figure. The significance of these results is discussed.

The total estrogens in the fluid recovered from the individual follicles was one-third as great in the non-estrous phases of the cycle as during estrus. However, the estrogen concentration per unit volume of fluid was constant throughout the cycle. The importance of the latter observation has been thoroughly discussed.

A total of 128 urine samples collected from 24 mares at 2 to 3-day intervals during the entire cycle were assayed by a chemical method for their total estrogen content.

Evidence indicating two peaks in estrogen excretion in the non-pregnant mare, one during estrous and one between the tenth and fifteenth day, has been presented. Citation has also been made of a marked rise in estrogen excretion by the pregnant mare beginning as early as 30 days after conception occurred.

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THE PRODUCTION OF OVULATION AND FUNCTIONAL CORPORA LUTEA IN RABBITS BEFORE AND AFTER TRANSECTION OF THE HYPOPHYSIAL STALK[1]

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TRANSECTION of the hypophysial stalk in the rabbit prevents the occurrence of mating induced ovulation and the associated corpus luteum formation (1). Failure of stalk-cut animals to ovulate is not due to an absence of ovulation producing material in the hypophysis. The glands of such animals contain normal concentrations of follicle stimulating and luteinizing substances (2). Additional evidence that the pars distalis retains some functional ability after transection of the hypophysial stalk is furnished by the fact that the ovaries do not become atrophic. Follicular development occurs to such a degree that ovulation can be produced readily by injections of pregnancy urine extracts (1).

Ovarian reactivity to the ovulation producing material of pregnancy urine decreases rapidly following removal of the hypophysis (3). White and Leonard (4) found that in order to induce ovulation in rabbits injected 1 hour after hypohysectomy it was necessary to use 50% more pregnancy urine than the minimal dose for a normal animal. Robson (5) showed that pregnancy urine extracts fail to produce ovulation in rabbits 3 days after hypophysectomy; injection of as much as 5 to 10 times the quantity of extract required for the production of ovulation in normal females proved ineffective. He suggested that in the rabbit the normal ovarian reactivity (i.e., the capacity of the ovary to respond by follicular growth, ovulation and corpus luteum formation to hormonal stimuli) is maintained by the constant secretion of small subthreshold doses of gonadotropic hormone from the anterior pituitary. Thus various lines of evidence indicate that the functional activity of the anterior lobe of the hypophysis before and after stalk transection can be tested by determining the quantity of pregnancy urine extract required to produce ovulation.

Deanesly, Fee and Parkes (6) found that although newly formed corpora lutea continue to develop for a few hours after hypophysectomy this growth is retarded and degenerative changes soon occur. Smith and White (7), Firor (8) and Foster, Foster and Hisaw (9) have shown that degeneration of the corpora lutea in the rabbit occurs very promptly following hypophysectomy. In this species, continuous anterior lobe activity is essential to the full development and maintenance of the corpus luteum function. It therefore occurred to us that additional information concerning anterior lobe function in the stalk-cut animal could be obtained by a study

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of the duration of activity of the corpora lutea formed as a result of injections of pregnancy urine extract.

METHODS

The pregnancy urine extract used was antuitrin-S (Parke, Davis & Co.). In attempting to determine the minimal quantity of extract necessary to produce ovulation we soon found, as did Zondek (10) and Zondek and Sulman (11), that animals rapidly become refractory to the extract. Consequently a closely graded series of injections could not be given to the same animal. By comparing the responses of 15 normal female rabbits we were able to determine the minimum quantity of extract which, with few exceptions, produced ovulation in normal females of our colony. In preliminary tests of the ovulatory response 6 stalk-cut females were injected with graded doses of antuitrin-S. Six additional rabbits were tested by single injections before and after transection of the hypophysial stalk. In the case of 6 normal and 6 stalk-cut rabbits similar tests were made with a second ovulation producing substance, mare serum extract (gonadogen, the Upjohn Co.). Gonadogen and antuitrin-S injections were given from 2 weeks to 5 months after stalk transection; in the majority of cases the tests were made one month after operation. Both extracts were administered intravenously. Laparotomies were performed on the third, fourth or fifth days following injection to determine whether or not ovulation and corpus luteum formation had occurred.

In addition to histological studies of the ovaries, progestational changes and decidual reactions of the uterus were used as criteria of corpus luteum function. Decidual responses were induced by passing a thread through the lumen of one uterine horn. Seven to 10 days later the segment of the uterus containing the thread was excised and fixed in formalin-Zenker fluid. This tissue was sectioned and stained with hematoxylin-eosin. The duration of corpus luteum function was estimated in the following way. Threads were placed in the uteri of a series of animals at intervals varying between 5 and 14 days after ovulation; if a decidual reaction occurred it was concluded that the corpora lutea were still functional.

The technic used in the transection of the hypophysial stalk was similar to that used previously (1). In every case histological examinations of the hypophysis and of the base of the brain were made to determine whether or not stalk section had been complete.

RESULTS

Single intravenous injections of 0.1 cc. antuitrin-S (10 R.U.) sufficed to cause ovulation in 12 of 15 normal animals. This amount of extract was considerably greater than the minimal ovulation producing dose of prolan as determined by Friedman (12) who reported that 1 R.U. of prolan per kg. of body weight was sufficient. White and Leonard (4) found that a total of 2 R.U. of prolan produced ovulation in some rabbits.

Three stalk-cut females ovulated in response to an initial injection of 0.1 cc. (10 R.U.) of pregnancy urine extract, but in 3 others, which likewise had not been injected before operation, abnormally large amounts of extract (1.0 to 1.5 cc.) were necessary. In these animals, which failed to ovulate when first injected, the ineffective doses may have increased refractoriness. Consequently the amounts of extract which finally produced ovulation may have been somewhat greater than the initial minimal dose.

In 6 additional females the ovulation threshold was determined before transection of the hypophysial stalk and the same minimal amount of extract injected one month after operation. Only one of these animals required more extract for the production of ovulation following the stalk transection. The ovaries of stalk-cut animals thus retain, to a large degree, their sensitivity to the ovulation producing substance of

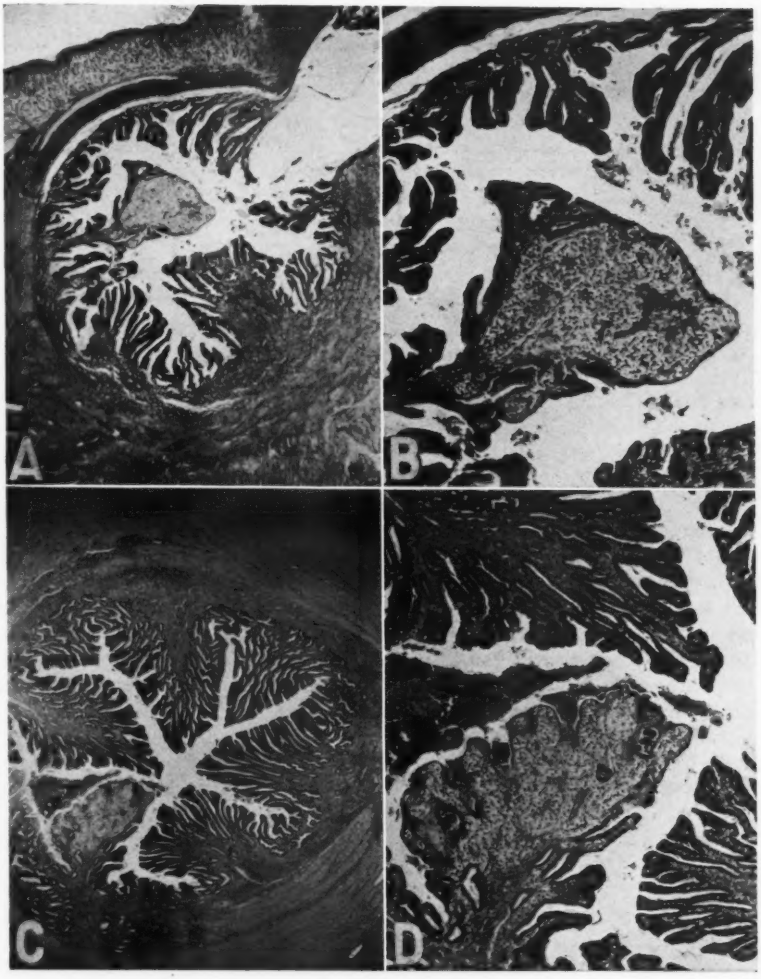

Fig. 1. THE UTERUS OF A NORMAL RABBIT (A and B) OF A STALK-CUT RABBIT (C and D) SHOWING PROGESTATIONAL PROLIFERATION AND A DECIDUOMA. Thread was placed in uterus 10 days after ovulation and the tissue removed one week later.

pregnancy urine. In this respect they differ greatly from hyophysectomized rabbits.

Similar results were obtained when the ovulation producing substance of mare serum was used. It was found that 0.6 U of gonadogen per kg. of body weight produced ovulation in 5 out of 9 normal females. In the remaining four 0.7, 0.8, 1.0 and 1.2 U per kg., respectively, were required. Of 6 stalk-cut females 3 ovulated when 0.6 U of gonadogen per kg. of body weight was given. One ovulated following an injection of 0.8 U and another when given 1.5 U. One rabbit did not ovulate when given as much as 2.0 U per kg.

These experiments have again demonstrated that transection of the hypophysial

stalk does not seriously impair the functional activity of the hypophysis. The ovaries of many stalk-cut rabbits are maintained in such a condition that ovulation occurs following injections of ovulation producing substances in quantities which are barely sufficient to produce ovulation in normal females. In 8 out of a total of 21 animals stalk transection did produce a deficiency in hypophysial function which rendered them refractory to prolan. Ovulation did not occur unless very large amounts of extract were administered. Histological examination of the pituitary glands of such animals revealed unmistakable signs of degenerative changes in the pars distalis. It was felt that in these cases hypophysial tissue had been injured directly or the blood supply of the gland had been greatly impaired by the operation.

In normal rabbits experimentally induced corpora lutea retain their activity for 14 to 16 days (13). For example, Loeb and Smith (14) reported that marked degeneration does not occur in the corpora lutea of non-pregnant rabbits until the eighteenth day. Loeb (15) obtained decidual responses in rabbits 10 days after ovulation and Friedman (12) found that these decidual reactions could not be induced on the fourteenth day following injection produced ovulation. In our series of 19 normal rabbits decidual reactions could be elicited from the fourth to the twelfth day after ovulation, but not later. The corpora lutea showed signs of retrogression after the fourteenth day in the 6 cases in which histological studies of the ovaries were made. Progestational changes occurred in each of the 12 stalk-cut rabbits which ovulated as a result of injections of pregnancy urine extract. Threads were placed in the uteri of 6 of these animals between the fifth and tenth day following ovulation; each gave a positive reaction. A thread inserted in the uterus of one rabbit on the eleventh postovulatory day induced a decidual response. In the remaining 5, decidual reactions failed to occur as a result of stimulating the uterus after the tenth postovulatory day. The corpora lutea of the stalk-cut animals likewise showed degenerative changes after the tenth or twelfth day. In most of the stalk-cut rabbits neither the progestational nor decidual responses were as great as those seen in normal animals.

DISCUSSION

Since corpora lutea fail to develop fully in animals hypophysectomized immediately after injection of prolan, and since fully developed corpora lutea degenerate rapidly following removal of the hypophysis, it appears that continuous anterior lobe activity is essential to the growth and function of the corpus luteum. The experiments reported here show that in stalk-cut animals corpora lutea may persist for as long a time as do the corpora lutea of pseudopregnant control rabbits. Further, these corpora lutea are capable of secreting sufficient hormone to produce progestational changes in the uterus and to so sensitize the uterine mucosa that a decidual reaction can be evoked. The hypophysis of the stalk-cut rabbit, therefore, must be capable of maintaining a normally functioning corpus luteum.

Progestational changes were found to have occurred in every case, normal and stalk-cut, in which the dose of antuitrin-S was sufficient to cause ovulation. In those animals in which only two or three corpora lutea had formed, the progestational changes were less marked and decidual reactions did not occur. This is in agreement with Friedman's (16) suggestion that more of the lutein hormone is required for uterine sensitization than is required for the proliferative changes.

In a few cases transection of the hypophysial stalk did cause deficiencies in anterior lobe function; abnormally large quantities of pregnancy urine extract were required for the production of ovulation. The ovaries and uteri were small and abnormal in appearance. Histological examination of the hypophysis gave further indication of a hypofunctional condition.

SUMMARY

Ovulation was produced in rabbits after transection of the hypophysial stalk by injections of quantities of ovulation producing extracts (antuitrin-S and gonadogen) barely sufficient to produce ovulation in normal control rabbits.

The corpora lutea formed in the ovaries of stalk-cut animals following injection of the ovulation producing extracts were functional. Progestational changes occurred in the uterus and decidual reactions could be evoked.

The corpora lutea of both the normal and stalk-cut rabbits remained functionally active for ten to twelve days following injection produced ovulation.

We wish to thank George F. Cartland of the Upjohn Company for supplying the gonadogen used in this work.

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A STUDY OF THE MECHANISM WHEREBY COPPER ACETATE AND CERTAIN DRUGS PRODUCE OVULATION IN THE RABBIT[1]

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THE RESULTS of various experiments performed in several laboratories indicate that the discharge of an ovulation producing substance from the pars distalis of the rabbit's hypophysis following coitus is neurally mediated (1). Ovulation can be evoked in the rabbit by electrical stimulation of various regions of the hypothalamus (2, 3). It has been reported recently that certain drugs and other chemical substances likewise produce ovulation in the rabbit.

Fevold, Hisaw and Greep (4) found that copper salts, when injected intravenously, cause rabbits to ovulate. They concluded that "Copper salts probably cause ovulation by increasing the effective activity of the gonad stimulating hormones present in the blood and thus the threshold for ovulation is attained." Bischoff (5), on the other hand, suggested that "since ovulation in the rabbit is apparently brought about by nervous stimulation, it should not be surprising that the injection of 10 mg. of as toxic a substance as copper acetate should lead to nervous stimulation and ovulation." Marshall (6) and Marshall, Verney and Vogt (7) reported that while injections of picrotoxin frequently induce ovulation in the rabbit certain other convulsant drugs fail to do so. Marshall, Verney and Vogt stated that "The most reasonable interpretation of our results, however, would appear to lie in a more elective action on the innervation of the anterior lobe of the pituitary by picrotoxin than by the other drugs whose action in this regard we have investigated."

These observations suggested an excellent means for testing the hypothesis that certain gonadotropic functions of the hypophysis can be neurally mediated. We felt that if the original conclusions of Fevold, Hisaw and Greep were correct, copper salts should produce ovulation on injection immediately after severance of all known nerve connections between hypophysis and central nervous system; it seemed reasonable to assume that the concentration of gonadotropic hormone in the blood would not be greatly altered by the operation. Support for this assumption is furnished by the fact that stalk transection does not reduce the gonadotropic hormone content of the anterior lobe of the gland (8). Similarly, if the ovulation producing action of picrotoxin were dependent on an anterior lobe innervation severance of these nerves should abolish the effect.

METHODS

The central connections of the sympathetic fibers which supply the hypophysial region were severed by removal of both superior cervical ganglia, and the more direct

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hypothalamico-hypophysial neural connections were interrupted by transection of the hypophysial stalk. As in previous work (9), a temporal approach to the stalk was employed. At autopsy the hypophysis and that portion of the hypothalamus adjacent to the gland were sectioned serially. Thus histological verification of the completeness of stalk section was obtained.

Since we wished, in some instances, to test the effect of hypophysial stalk section performed just before or just after injection of a copper salt it was necessary to ascertain whether or not an anesthetic would modify the copper induced ovulatory reaction. Therefore, 6 normal animals were anesthetized with nembutal (45 mg. per kg.) before or shortly after administration of the copper salt.

A 1% copper acetate solution was employed and the dose given was 10 or 15 mg. of the salt per individual. The dose of picrotoxin used was 0.5 to 1.5 mg. per kg. of body weight. In those instances in which picrotoxin convulsions were severe, small amounts (32 mg.) of nembutal were given to quiet the animals. Other details concerning the drugs used, dosages and effects will be given as the results obtained are described.

On the third or fourth day following injection, the animals were anesthetized and laparotomies performed. The presence of newly formed corpora lutea in the ovaries was considered an indication that an anterior lobe discharge sufficient to evoke ovulation had occurred.

It was felt that negative results obtained in many of the preliminary experiments with picrotoxin and other drugs might have been due to lack of fully mature ovarian follicles in the test animals. Even during the most favorable seasons of the year a few females are probably not in an optimal condition for ovulation to occur. An effort was made to induce follicular development in 6 normal and 4 stalk-cut rabbits by subcutaneous injections of an FSH preparation[2] in amounts (5 R.U. daily for 3 consecutive days) which do not produce ovulation. In the majority of cases, however, the rabbits used were untreated normal females which had been isolated for several weeks.

RESULTS

Copper acetate. The dosage used was near the lethal level and a few animals died as a result of the injection. Rabbits in which convulsions occurred invariably died. In all cases there was cyanosis, and acceleration of respiration, restlessness and slight salivation. These symptoms were more pronounced in some animals than in others. Normal females which survived the injection invariably ovulated. Surgical anesthesia induced by nembutal did not abolish the ovulatory effect of copper acetate. The injection of this salt caused rabbits to ovulate even after removal of both superior cervical ganglia, but in every case transection of the hypophysial stalk prevented ovulation (table 1). When the stalk was cut just before or just after injection ovulation did not occur. The combination of operation, anesthetic and toxic salt proved fatal in many instances and approximately one-third of the animals in which stalk section was performed shortly after copper acetate had been injected died within 10 to 12 hours. Failure of ovulation to occur in such cases was not regarded as significant. A few control operations were performed; the stalk was exposed but not severed. In these rabbits anesthesia and intracranial trauma did not abolish the copper induced ovulation. The only animals which ovulated after stalk transection were those which had been injected 2 hours or more before operation. It was found by Fee and Parkes (10) that the entire pituitary gland could be removed 1 hour after coitus without preventing ovulation. Earlier removal abolished the response. Either copper acetate

[2] The FSH preparation used was kindly furnished by the Squibb Institute for Medical Research.

TABLE 1. PRODUCTION OF OVULATION IN THE RABBIT BY INTRAVENOUS INJECTIONS OF COPPER ACETATE

No. of animals	Condition of animals	Body wt., ave. and range	Total amount injected	Ovulation
		kg.	*mg.*	
6	Normal	3.13 (2.50–3.37)	10 or 15	+
6	Normal anesthetized	2.31 (2.04–2.50)	15	+
8	Stalk-cut 30 days or more	2.98 (2.50–3.30)	15	−
5	Stalk-cut 1 to 3 days	2.93 (2.55–3.55)	15	−
6	Stalk-cut 10 to 30 minutes	2.27 (2.02–2.62)	15	−
6	Stalk-cut after the injection 10 to 90 min.	2.39 (2.10–2.60)	15	−
2	110 to 300 min.	2.10 & 2.45	15	−
6	110 to 300 min.	2.41 (2.07–2.65)	15	+
5	Control operations	2.62 (2.25–2.80)	15	+
4	Bilateral removal of superior cervical ganglia	2.29 (1.75–3.03)	15	+

requires a longer time for its action on the hypophysis or the combination of trauma and anesthesia stops the chemically evoked ovulatory process unless it is well advanced. Marshall, Verney and Vogt (7), in their study of artificially induced ovulation, found that the period between chemical stimulation and ovarian reaction is very much longer than that intervening between coitus or electrical stimulation and ovulation.

Picrotoxin and metrazol. We were able to confirm the observation (6) that intravenous injections of picrotoxin cause ovulation in certain rabbits. A few animals showed no detectable ovarian response and in others follicular enlargement appeared to be the only effect occurring. Presumably, those rabbits which failed to respond to this drug were not in full estrus at the time of injection. The administration of small amounts of an FSH extract before injection of the drug did not increase the proportion of positive responses. It can not be assumed, however, that such injections reproduced all the phenomena of normal estrus. The small doses of nembutal given in some instances to minimize convulsive seizures may have inhibited ovulation in a few cases but several anesthetized rabbits ovulated. Two of four rabbits injected after bilateral ablation of the superior cervical ganglia ovulated. Eight chronic stalk-cut animals and 3 in which the stalk had been transected 2 to 3 hours before injection failed to ovulate when corresponding amounts of picrotoxin were administered even though equally intense convulsions ensued (table 2).

TABLE 2. PRODUCTION OF OVULATION IN THE RABBIT BY INTRAVENOUS INJECTIONS OF PICROTOXIN

No. of animals	Condition of animals	Body wt., ave. and range	Amount injected	Ovulation
		kg.	*mg./kg. body wt.*	
9	Normal	2.39 (1.55–3.45)	0.8 to 1.2	−
4	Normal	3.26 (2.70–3.77)	0.8 to 1.2	±
5	Normal	2.89 (2.32–3.27)	1.0 to 2.0	+
8	Stalk-cut 30 days or more	2.83 (2.23–3.65)	0.8 to 1.5	−
3	Stalk-cut 2 to 3 hours	2.74 (2.23–2.50)	1.0 to 2.0	−
2	Bilateral removal of superior cervical ganglia	2.25 and 2.52	1.5	+
2	Bilateral removal of superior cervical ganglia	2.70 and 2.80	1.5	−

± = Corpora hemorrhagica or abnormally large follicles.

Metrazol (50 to 100 mg. per kg. subcutaneously or 20 to 40 mg. per kg. intravenously) caused salivation, vasoconstriction, respiratory changes, long continued

restlessness and convulsions. Four rabbits ovulated as a result of such injections and 3 showed slight enlargement of follicles. Nine others which had similar somatic reactions failed to show any detectable ovarian response. Metrazol appears to be even less effective than picrotoxin. Five stalk-cut rabbits failed to ovulate following injection of the drug (table 3). Although metrazol, in amounts equal to or greater than the doses which caused some normal females to ovulate, failed to produce ovulation in any of the stalk-cut animals we feel that the experiments are too few in number to be significant. Since ovulation occurred so infrequently in normal animals it seemed inadvisable to pursue the matter further.

TABLE 3. PRODUCTION OF OVULATION IN THE RABBIT BY MEANS OF METRAZOL INJECTIONS

No. of animals	Condition of animals	Body wt., ave. and range	Amount injected	Ovulation
		kg.	*mg./kg. body wt.*	
9	Normal	2.04 (1.53–2.70)	20 to 100	−
3	Normal	2.25 (2.00–2.50)	50 to 100	±
4	Normal	1.92 (1.55–2.30)	60 to 100	+
5	Stalk-cut 30 days or more	2.59 (1.75–3.50)	60 to 120	−

± = Corpora hemorrhagica or abnormally large follicles.

Convulsive drugs. Strychnine sulfate injected subcutaneously into 5 rabbits anesthetized with nembutal did not produce ovulation. Sufficient quantities of the drug were given (0.4 to 2.0 mg. per kg.) to cause hyperirritability in all instances and actual convulsions in most cases. Repetitive mechanical stimulation of the vulva and vagina by means of a glass rod 4 mm. in diameter during this period of heightened excitability likewise proved to be ineffective.

Doses of insulin which caused convulsions failed to elicit any ovulatory response in the 8 normal, mature, female rabbits used even though the convulsive seizures were preceded and followed by long periods (30 to 90 minutes) of restlessness and excitement.

Convulsions and general excitement were evoked in 7 normal animals by subcutaneous injection of benzedrine in doses of 2 to 10 mg. per kg. but in no case did ovulation result. One additional rabbit was treated with small amounts of follicle stimulating substance previous to injection, but laparotomy 4 days later revealed that benzedrine had not induced ovulation despite the presence of large follicles in both ovaries. Rabbits invariably died when more than 10 mg. per kg. of the drug were given.

The ovaries of 8 rabbits which had been injected subcutaneously with ephedrine hydrochloride (15 to 150 mg. per kg.) also showed no ruptured follicles or corpora lutea. One ovary of an additional rabbit which had received 150 mg. of ephedrine and had exhibited prolonged restlessness and signs of general excitement contained a hemorrhagic follicle. Since corpora hemorrhagica are occasionally found in normal unmated and untreated rabbits we attach no significance to this observation.

DISCUSSION

Copper salts, picrotoxin and metrazol do cause ovulation in the normal estrous rabbit. The two drugs are much less effective than copper acetate and ovulation does not occur unless conditions are favorable. Many other drugs and materials capable of causing convulsions or symptoms indicative of central nervous system excitation do not initiate an ovulatory response in the rabbit. It is conceivable that other drugs, convulsants or toxic materials could be found which would cause ovulation through

some direct or indirect action on the mechanism which controls the ovulation producing function of the hypophysis. Many materials, however, have been found to be ineffective (7, 9, 11).

The suggestion of Fevold, Hisaw and Greep (4) concerning the action of copper salts is a very reasonable one and has much to recommend it. The experiments described here, however, do not support this hypothesis. They indicate, rather, that copper acetate acts on a central nervous mechanism to initiate discharge of an ovulation producing substance from the hypophysis. The experiments performed with picrotoxin and metrazol, though less convincing, also furnish results in agreement with the hypothesis that these materials act through the central nervous system. We are unable to explain just why these three quite dissimilar substances cause ovulation in the rabbit when other excitants and convulsants apparently fail to do so.

SUMMARY

Ten or fifteen mg. of copper acetate injected intravenously invariably produced ovulation in normal unanesthetized rabbits, in normal rabbits anesthetized with nembutal and in animals which had been subjected to bilateral ablation of the superior cervical ganglia.

Rabbits in which the hypophysial stalk had been transected 30 days or more previously failed to ovulate as a result of copper acetate injections.

Transection of the hypophysial stalk immediately before injection of copper acetate or at any time during the following 90 minutes likewise prevented ovulation.

Convulsive doses of picrotoxin and metrazol induced ovulation in not more than half of the normal rabbits injected. Stalk-cut preparations failed to ovulate when comparable amounts of these drugs were administered.

Strychnine sulfate, insulin, benzedrine and ephedrine, though given in large quantities, failed to initiate ovulation.

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ABNORMAL GROWTH IN A STRAIN OF RATS CHARACTERIZED BY LOW FERTILITY AND A HIGH INCIDENCE OF BENIGN MAMMARY TUMORS[1]

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RECENTLY (1) we described the Albany (A-S) strain of rats in which there is a relatively high incidence of mammary adenofibromata among the females. In certain lines approximately 40% of the females 14 months of age and above develop mammary tumors. The fertility of this strain of rats is fairly low and is caused, in part at least, by three spontaneous abnormalities of the reproductive organs. First, the germinal epithelium of many males over 10 months of age undergoes degeneration. Second, many of the females exhibit an ovarian dysfunction characterized by complete or partial failure of ovulation and subsequent follicular atresia or direct luteinization (2). As a result, prolongation of the follicular phase of the estrous cycle with either partial or complete vaginal cornification occurs and approximately 30% of all females never become pregnant. Third, approximately 25% of the females that do become pregnant exhibit prolonged vaginal bleeding during pregnancy which is associated with resorption of some or all of the fetuses (3). We have previously compared the abnormalities in the female rats with those which follow complete and incomplete hypophysectomy and have suggested that the A-S rats present an inherent dysfunction of the anterior pituitary (2).

It has also been observed that these animals exhibit subnormal growth. Since growth, as well as reproductive function, is largely determined by the anterior pituitary, this finding has been considered to have some bearing on the possible pituitary dysfunction just mentioned. In the present communication the growth of this strain and that of a "control" strain (V-S) maintained under identical conditions is analyzed. The growth of the V-S strain is excellent, being somewhat superior to the standard given by Donaldson (4). In contrast to the A-S strain, the fertility is high and the tumor incidence comparatively low.

In both the A-S and the V-S colonies all females, as well as a certain percentage of males, were routinely weighed at 10-day intervals from the time of weaning (25 days) until they were 100 days old; they were then weighed at monthly intervals until death. Although they were constantly with males, approximately 30% of the A-S females were sterile; therefore it was possible to compare the growth of breeding and non-breeding rats of this strain. Judging by the absence of sperm in the vaginal smears, it is probable that a majority of the latter rats were virgins. Comparable studies were carried out in the V-S strain by comparing the growth of breeding and virgin females. However, it should be emphasized that the V-S virgins were not strictly comparable to the A-S non-breeding females, since the latter were physio-

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logically sterile, while the V-S virgins, judging by past observations, were potentially fertile. In addition, studies on the comparative growth of A-S and V-S males were made.

RESULTS

The pertinent findings of this study are shown in figures 1 and 2. These figures are based on weights made on the following groups of rats: (*a*) 109 A-S females which had delivered young, (*b*) 52 A-S females which failed to reproduce, (*c*) 52 V-S breeding females, (*d*) 59 V-S virgin females, (*e*) 84 A-S male rats and (*f*) 47 V-S male rats. Tumors were not found in any of these rats. Weights were obtained in each case until the death of the animal, which in a few instances did not occur until approximately 800 days. However, since the death rate of the A-S rats began to rise

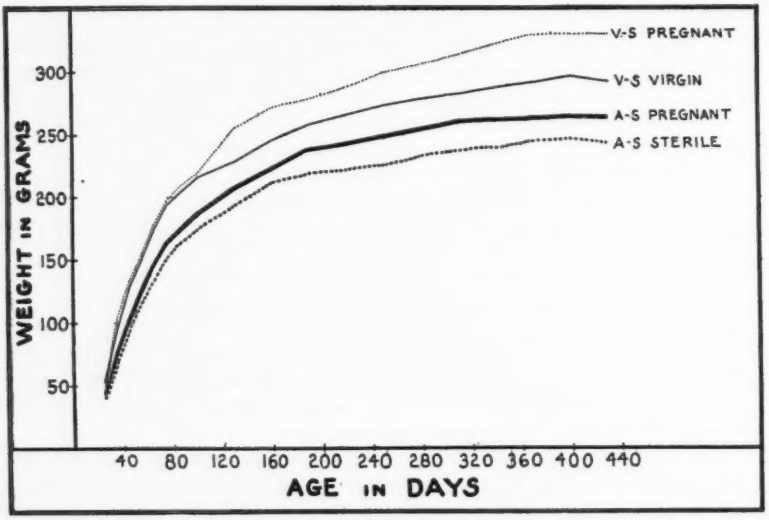

Fig. 1. Comparative growth in A-S breeding and non-breeding female rats and the V-S breeding and virgin rats.

fairly rapidly after 440 days, the growth curves are carried only to this point. The growth curves of A-S female rats with tumors, both breeding and sterile, were also studied. They practically coincided with those of comparable groups of A-S rats free of neoplasms and are not presented here.

Study of figure 1 indicates that the breeding and sterile female A-S rats were distinctly inferior to the breeding and virgin V-S rats in weaning weight, growth rate and final weight. Figure 2 indicates that the A-S males were inferior to the V-S males in the same respects. In order to ascertain if the differences in growth between the two strains were statistically significant, comparison was made of average weights attained by (*a*) the A-S and the V-S breeding animals at 400 days of age, (*b*) the A-S sterile animals and V-S virgins at 340 days and (*c*) the A-S males and the V-S males at 440 days of age. It was found that the differences in weight between the comparable groups in the two strains were highly significant (table 1).

In both the A-S and V-S female groups the breeding females grew more rapidly and attained a higher final weight than did the non-breeders (fig. 1). In the A-S

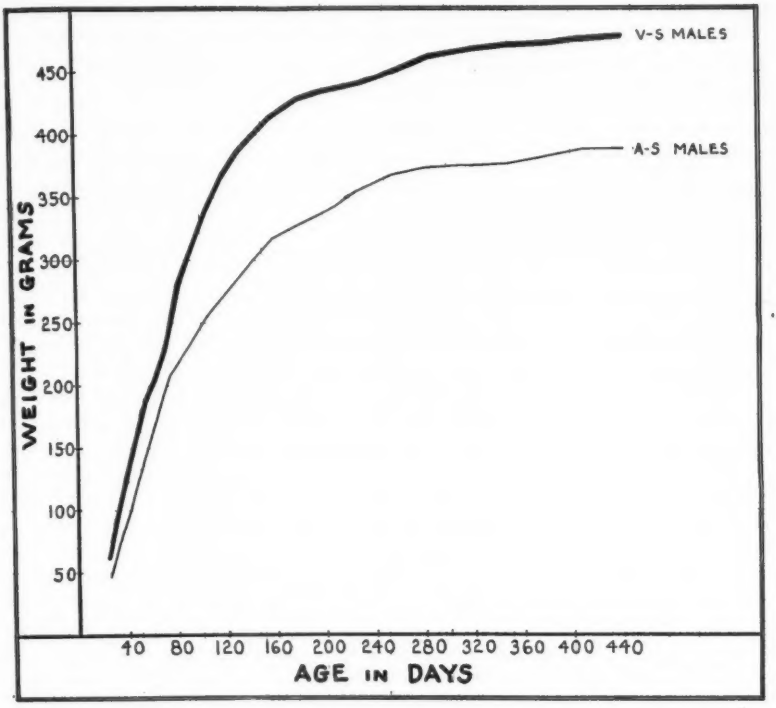

Fig. 2. Comparative growth in the A-S and V-S male rats.

groups it was found, moreover, that there was considerable tendency for the females, which subsequently became pregnant, to exhibit superior growth even before the first pregnancy (fig. 1). Therefore, the weights of the future A-S breeders were compared with those of the future A-S non-breeders at 65 days of age. The weight differences between the two groups at this early age were statistically significant. This

Table 1. Comparison of body weights attained by A-S and V-S rats

Group	Number of rats	Ave. No. of pregnancies	Age	Mean wt. ±P.E.	Diff.±P.E.	Diff.[1] / P.E. diff.
			days	*gm.*		
V-S (Breeding)	12	7	400	329± 10.0	64± 10	6.4
A-S (Breeding)	57	4	400	265± 0.99		
V-S Virgin	24	—	340	288± 3.4	51± 4.9	10.4
A-S Sterile	21	—	340	237± 3.6		
V-S Male	33	—	440	478± 4.6	90± 7.14	12.6
A-S Male	21	—	440	388± 5.4		

[1] When the value is 3 or over, the difference between the two means is considered significant.

finding definitely associates inferior growth with subsequent failure to reproduce. In contrast, there was no difference in the growth rate of the breeding and virgin V-S females until the animals assigned to the breeding group actually became pregnant; from this point onward the litter bearers exhibited superior growth.

DISCUSSION

These observations indicate two facts. First, rats belonging to the A-S strain exhibit distinctly inferior growth when they are compared with similar groups of V-S rats. Second, in both strains the breeding females grow faster and attain a higher body weight than do those which do not bear young. The latter finding, particularly in the case of the V-S rats where the experimental conditions were similar, confirms the results of several investigations, including those of Cole and Hart (5) and Bogart, Sperling, Barnes and Asdell (6) who reported that pregnancy stimulates growth in the rat. The former workers found pregnancy to be associated with increased food intake which was considered to be possibly due to release of certain anterior lobe factors. The latter workers recalled the fact that injection of estrin inhibits growth in the rat and suggested the possibility that the condition of pregnancy held in abeyance the growth inhibiting properties of estrin produced by the animal itself.

Observations made on the V-S rats lend support to the concept of Bogart and his associates. Vaginal smears were made daily on all rats from early in life until death. From these data the exact proportion of the observation period that each animal was pregnant, lactating, or in the various phases of the unmated cycle was calculated. The vaginal smears of the V-S virgins were made up of either epithelial or cornified cells, or a mixture of these two, for 45% of the almost life-long observation period. Since the finding of such cells in the smear is indicative of the presence of estrin, it may be assumed that at least vaginal threshold amounts of estrin were operative in these animals for this portion of the observation period. On the other hand, the group of V-S breeders presented such smears for only 13.9% of the observation period and were either pregnant or lactating for approximately 65% of this time. Since administration of estrin is known to inhibit growth (7), the association of the inferior growth of the virgin rats and the extended period during which the action of estrin was manifested in these animals seems significant. If, as is quite possible, a high level of estrin is operative also during pregnancy in the rat, the findings of Allen and Meyer (8), which indicated that progestin restricted the action of estrin on the vagina, might be of significance. The active corpora lutea of pregnancy and lactation in the V-S breeders may have nullified the growth inhibiting properties of estrin, a possibility already suggested by Bogart and his associates (6). However, we do not know of experiments in which this effect of progestin has actually been demonstrated.

The reason for the inferior growth of the A-S rats as compared with that of the V-S rats is obscure. Since growth, as well as reproductive function, is under the control of anterior pituitary activity, the poor growth in the rats might be considered another indication of anterior lobe dysfunction in the A-S strain. If it is actually proven that these rats suffer from a spontaneous pituitary dysfunction, they should serve as excellent test animals for studying the effects of certain anterior lobe factors because of the fact that the spontaneous deficiency more nearly simulates conditions in man. It should be admitted, however, that we have no clear-cut evidence that a decreased activity of the anterior lobe is responsible for these abnormalities.

The fact that the A-S sterile females exhibited relatively poorer growth than the A-S breeding rats cannot be satisfactorily explained by their failure to become pregnant since statistically significant weight differences were noted between the two groups before the rats were actually mated. It would seem that the combination

of complete sterility and poorest growth found in this particular group represented the most severe manifestation of the dysfunction, whatever its nature, which occurred to a lesser degree in the colony as a whole.

It would seem desirable to ascertain if the growth and reproductive abnormalities found in the A-S strain are correlated with structural changes in the endocrine glands, particularly the anterior pituitary. Therefore, a comparative cytologic study of the anterior pituitary in the A-S and V-S rats has been initiated. In this connection recent observations (9) on the anterior pituitaries of different strains of inbred guinea pigs are pertinent. It was found that a strain which exhibited comparatively inferior growth presented statistically lower levels of eosinophils in the anterior pituitaries than were found in a strain which grew more rapidly and attained a higher final weight.

SUMMARY

The A-S strain of rats is characterized by spontaneous reproductive disorders and a relatively high incidence of benign mammary tumors in the older females. Weaning weight, rate of growth and final weight in both the male and female rats of this strain were decidedly inferior to that found in a control V-S strain in which fertility is excellent and the incidence of mammary tumors comparatively low. In both the A-S and V-S rats the breeding females grew more rapidly and attained a greater weight than did the non-breeding animals. The possible hormonal factors concerned with the inferior growth in the A-S strain are considered.

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EVIDENCE FOR THE PRESENCE OF A SECOND MAMMOGENIC (LOBULE-ALVEOLAR) FACTOR IN THE ANTERIOR PITUITARY[1,2]

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LEWIS AND TURNER (1) have reviewed the evidence for the non-effectiveness of ovarian hormones in stimulating mammary gland hyperplasia in the hypophysectomized animal. The results of a number of such experiments, reported since 1935, have been uniformly negative. It has been shown that the mammary gland can be developed in hypophysectomized animals with injections of anterior pituitary from pregnant animals or from animals given preliminary treatment with estrone (1–6). Guinea pigs, rabbits, rats and mice have been employed in such studies. Many experiments had shown that estrogen will grow the mammary ducts in most species, whereas à combination of estrogen and progestin is required to cause complete lobule-alveolar proliferation (see Turner, 7, for review). When it was established, in this laboratory, that the ovarian hormones act on the AP causing development there of a mammary growth factor (1, 2), it seemed probable that the mammogenic growth complex consisted of a duct growth and a lobule-alveolar growth factor (3). Evidence to support this supposition has now been obtained.

The presence of an AP duct growth factor (mammogen 1) has been established. Lewis and Turner (1) showed in an extensive series of experiments that fresh AP and extracts would grow the mammary duct systems of male and castrate female mice and rabbits and hypophysectomized ground squirrels. A large series of anterior pituitaries from cattle were assayed using male mice and the content of mammogen 1 was found to agree well with the known facts of mammary growth. This factor was highest during the early estrual cycles of heifers. During pregnancy the AP content of mammogen 1 was found to increase to a peak at about mid-pregnancy, when mammary hyperplasia is at its maximum, and then declined. The AP of lactating cows contained more than did the AP of dry cows and dairy cows more than beef cows.

Estrone was found to increase the AP content of mammogen 1 in male rabbits to more than the content found in pregnant females. Extracts were prepared which had 400 times the potency of the fresh AP, comparing extracts in oil to fresh AP in water.

Some evidence for the presence of two mammogenic factors was also presented, as fresh AP was found to produce lobule anlage in castrate female mice within 6 days' injection whereas potent duct growth extracts failed on long time administration. The present report confirms and extends the evidence for the presence and action of a second mammogenic AP factor.

PROCEDURE

Young virgin female mice weighing from 15 to 20 gm. were castrated and a control mammary gland removed. If the control gland showed more than a system of smooth

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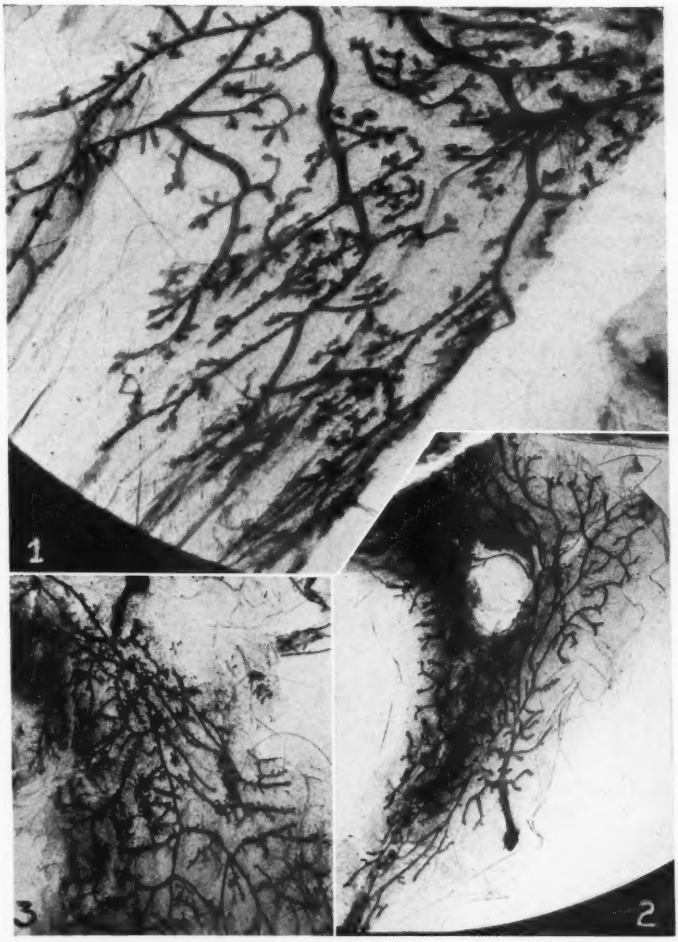

Fig. 1. MAMMARY GLAND FROM A MOUSE PREGNANT 4 DAYS (12×). Fig. 2. MAMMARY GLAND FROM A NORMAL, VIRGIN FEMALE MOUSE (4.7×). Fig. 3. MAMMARY GLAND FROM A YOUNG VIRGIN SPAYED FEMALE MOUSE GIVEN A TOTAL OF 75 MG. OF FRESH ANTERIOR PITUITARY LOBE TISSUE SECURED FROM PREGNANT CATTLE IN 5 DAYS (5×).

ducts after fixing and staining, the animal was discarded. The castrates were allowed several days to recover from the operation and to deplete the body of sex hormones. They were then injected once daily subcutaneously with fresh AP in water, or an AP extract. They were killed 24 hours after the last injection, their skins were removed and fixed in Bouin's solution, the subcutaneous fascia removed, stained in Mayer's hematoxylin and the mammary glands examined in acid alcohol under the dissecting microscope. Individual glands were mounted in clarite for preservation.

Examination of glands from pregnant female mice established that the first easily detectable signs of lobule formation consisted of the proliferation of numerous side stubs and flowery ends on short intralobular ducts (fig. 1). This stage was taken as the

minimum development for positive results. It is similar to the condition present at 4 days of pregnancy. It is not present in normal female mice which have never been pregnant or pseudopregnant (fig. 2).

RESULTS

Six groups of four mice each were given from 75 to 200 mg. of fresh AP from pregnant cattle in 4 days (table 1). At 75 mg. no pre-lobule response was obtained. In 3 groups given 100 mg. each, 5 mice showed the development of lobules. One of these was especially well developed. Three of four mice given 150 mg. each showed lobules but only one of a group given 200 mg. responded. Where lobule development did not occur enlarged end-buds and other signs of duct stimulation were usually present.

TABLE 1. LOBULE RESPONSE OF CASTRATE FEMALE MICE TO FRESH AP FROM PREGNANT CATTLE

Preparation	Days injected	No. mice	Dosage, mg.	Results	% Positive
Fresh AP from pregnant cows Lot V	4	4	75	0+, 4−	0
	4	12	100	5+, 7−	42
	4	4	150	3+, 1−	75
	4	4	200	1+, 3−	25
	5	2	75	1+, 1−	50
	5	4	100	1+, 3−	25
	5	4	150	2+, 2−	50
	5	4	200	3+, 1−	75
	6	12	50–70	0+, 12−	0
	6	4	75	3+, 1−	75
	6	10	100	5+, 5−	50
	6	6	120	5+, 0−	100
	6	5	135	5+, 0−	100
	6	9	150	5+, 4−	57
	6	6	165	6+, 0−	100
	6	7	180	6+, 0−, 1?	100
	6	5	200	3+, 2−	60
	8	4	50	0+, 4−	0
	8	4	75	0+, 4−	0
	8	4	100	2+, 2−	50
		114		57+, 57−	

Four groups of 4 mice each were given from 75 to 200 mg. of fresh AP in 5 days. Only 2 females survived in the group given 75 mg. One had responded with pre-lobule development (fig. 3). At 100, 150 and 200 mg. each, 1, 2 and 3 mice responded with early lobule development.

Eleven groups of castrate females were given from 50 to 200 mg. each in 6 days. Groups were composed of 4 to 10 mice. Fairly satisfactory results were secured although no smooth curve of response resulted perhaps because of too few mice per group. No positive response was obtained until a dosage of 75 mg. per mouse was reached when 3 out of 4 mice responded. Of 10 mice given 100 mg. each, half were found to have developed lobules. Responses to 120 and 135 mg. dosages were 100% but of a group of 9 given 150 mg. only 5 responded. Groups given 165 mg. and 180 mg. again gave 100% response but only 3 of 5 mice given 200 mg. each had lobules. Of the 64 mice in this study 39 developed lobules resembling the condition present from 4 to 10 days of pregnancy. In some cases very small glands were found to be heavily lobulated.

In 3 groups of 4 mice each, given 50, 75 and 100 mg. per mouse of fresh AP in an

8-day injection period only 2 mice, in the last group, responded with lobule development. The 8-day period did not appear to have any advantage over 6 days' injections. This study of the influence of the length of the injection period on lobule-alveolar growth, therefore, was not carried further.

An attempt was made to complete a graded curve of response to varying dosages of AP material with a 6-day injection period, but the material appeared to have deteriorated in lobule potency. For example, of 12 mice given 100 mg. only one developed lobules while all of 12 mice given 120 mg. and mice given 130 mg. were negative. Not until the dosage was raised to 285 mg. per mouse was a good response obtained.

In contrast to the excellent response to fresh AP, in only 2 isolated cases were lobules obtained out of 56 mice treated with mammogenic duct growth extract. This material (45) was obtained by warm ether-alcohol extraction of fresh AP from pregnant cattle and ether fractionation of the ether-alcohol soluble residue. It required only 0.23 mg. for one duct growth mouse unit (1).

To determine the ability of extract 45 to develop lobules in young castrate female mice, groups were given total dosages of 0.25, 0.5 and 1.0 mg. per mouse in 6 days. One response was obtained in the first and third groups (table 2). The same dosages

TABLE 2. LOBULE RESPONSE OF CASTRATE FEMALE MICE TO AP EXTRACT RICH IN THE DUCT GROWTH FACTOR

Preparation	Days injected	No. mice	Total dosage, mg.	Results
Mammogenic duct growth extract 45	6	3	0.25	1+, 2−
	6	4	0.50	0+, 4−
	6	3	1.00	1+, 2−
	6	6	0.25	0+, 6−
	6	6	0.50	0+, 6−
	6	6	1.00	0+, 6−
	14	6	0.70	0+, 6−
	14	6	1.40	0+, 6−
	14	6	2.80	0+, 6−
	13	3	4.8	0+, 3−
	24	4	9.2	0+, 4−
	30	3	39.2	0+, 3−
		56		2+, 54−

were repeated in groups of 6 mice with entirely negative results. We were at that time perfecting the technic of removing and examining the control glands. It is quite possible that glands from 2 previously pseudopregnant mice had evaded elimination.

Three groups of 6 mice were also administered extract 45 for 14 days being given total dosages of 0.7, 1.4 and 2.8 mg. per mouse. The results were entirely negative for lobule stimulation but many end-buds and other signs of duct stimulation were present in most cases. This was also true of the first groups reported above.

Another group of 10 castrate female mice given extract 45 also showed no lobule development. The dosage was 0.2 mg. per mouse for 2 days. This was then raised to 0.4 mg. a day. Three of the group were killed after 13 injections (4.8 mg. total injection) and 4 more after 22 days (8.8 mg. total injection) and the last 3 mice of the group were then given 5.0 mg. a day for 6 more days making a total of 39.2 mg. per mouse. Mammary duct systems were extensively developed but had no lobule hyperplasia.

DISCUSSION

Many experiments have shown that in most species of laboratory animals the injection of estrogen will cause hyperplasia of the mammary gland duct system while

a combination of estrogen and progestin is necessary for complete mammary gland growth. Subsequently it was discovered that the action of estrogen and progestin was indirect, their action being on the anterior pituitary which acts directly to cause mammary gland growth. It seemed probable then that there were two mammogenic factors in the AP, one which stimulated duct growth and the other which stimulated lobule-alveolar growth.

The experiments reported here confirm and extend the claims that lobule growth in castrate female mice has been secured with the subcutaneous injection of AP material from pregnant cattle. Development of the mammary gland to an extent approaching that of full pseudopregnancy was obtained in some cases where the AP material was administered for long periods of time with large dosages or when the mice responded especially well. The mammary lobule-alveolar growth of castrate female mice was much more uniform per mouse than in the case of duct growth in male mice. Where in the male one or several glands may respond with variations from negative to + 4 response, in the female all glands of one individual were found to be in practically the same state of lobule development.

It was not considered practical to require full pseudopregnant development as the criterion for a positive lobule-alveolar response. A stage of development was selected which shows an easily detectable development over that present in normal young virgin mice. This minimum development consists of the proliferation of many small interlobular ducts along the main ducts. This stage of development rarely resulted in these mice from the administration of mammogenic duct growth extract.

It appears that a separation of the two mammary growth factors of the AP has been effected. Only mammogen 1 (the duct growth factor) is present in ether-soluble extracts of the AP. Work is now progressing on the concentration of mammogen 2, the lobule-alveolar growth factor, and also on the perfection of a suitable technic for the assay of this hormone.

SUMMARY

Proliferation of prelobules was apparent after as few as 4 daily injections of AP from pregnant cows in castrate female mice. AP tissue was given to 114 such mice for periods varying from 4 to 8 days. Total dosages ranged from 50 to 200 mg. per mouse. Fifty-seven of these mice showed development of the mammary glands to the prelobule stage. They were equivalent to glands from mice pregnant 4 to 8 days.

An ether-soluble extract of cow AP (45) required only 0.23 mg. for one mouse unit of the duct growth factor. Fifty-six castrate female mice given from 0.25 to 39.2 mg. of this extract in 6 to 30-day injection periods showed almost uniformly no development of lobules although duct development was extensive in most cases. In 2 isolated cases early lobule development was apparent on autopsy. These positive results could not be repeated on trial.

Both the mammogenic duct growth and the lobule-alveolar growth factors are present in the fresh pregnant AP tissue of cattle. The mammogenic lobule-alveolar growth factor is apparently not associated with ether-soluble duct growth extracts of the AP at least as observed in experiments on the mouse.

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STUDIES ON THE RELATIONSHIP OF SEX HORMONES TO INFECTION

IV. A STUDY OF THE DISPERSION OR CONCENTRATION OF INDIA INK IN SKIN BY CERTAIN SEX HORMONES[1,2]

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IN RECENT YEARS it has become increasingly evident that sex and certain endocrine states play an important rôle in the host's response to disease incitants. Schultz (1) in 1934 reported that factors associated with sexual maturity in females reduces their reactivity to serum. Recently von Hamm and Rosenfeld (2) have reported that theelin affects antibody formation in pneumococcus infections.

In preceding papers we have shown that pregnancy (3) pseudopregnancy (4) and the estrogenic hormone (5) modify the rabbit's response to viral infection. It was also found that there is a close correlation between the spread of India ink in the skin and the rabbit's susceptibility to infection. This is in line with the findings of Duran-Reynals (6). In these papers we have extensively reviewed the literature and these references will not be repeated here.

It was found early in the course of this work that extreme variations occurred in so-called normal rabbits both in the spread of India ink and in the susceptibility of the animals to infection. Therefore a more thorough study of this subject seemed warranted. The India ink method was adopted since it is possible to make multiple determinations with India ink without modification of the animal's response and only a single determination with a virus or other infectious agent.

METHODS AND MATERIALS

Animals. The adult rabbits used in these experiments weighed between 2 and 3.5 kg. The immature rabbits weighed about 1 kg.

Standardization of condition. The animals were fed on a diet of Purina Rabbit Chow and hay, with free access to water. In order to avoid wide variations in the spread of India ink, it was found necessary first, to exclude all animals which had too great a variation from the other animals; second, to have at least 6 animals in each group studied as it was found that the individual rabbit even under the best conditions varied considerably from time to time. With as small a group as 6 rabbits, however, the variation of the mean could be kept below 10%. In order to avoid the effect of age or other factors as yet unknown the control groups and the experimental group were compared in the following manner: (*a*), the spread of India ink in the control group was not significantly different at the beginning and end of the experiment; (*b*), there was no significant difference in spread between the control group at the beginning and end of the experiment and the experimental group at the beginning of treatment; (*c*), the control group at the beginning and end of the experiment and the experimental animals before beginning treatment vary significantly from the experimental group after treatment. The results of these comparisons may be seen in table 2.

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[1] Read at the twenty-fourth annual meeting of the Association for the Study of Internal Secretions, New York City, June, 1940.

[2] Aided in part by a grant from the Duke University Research Council.

TABLE 1. THE SPREAD OF INDIA INK IN THE RABBIT'S SKIN

	No. of animals	Immediate			1 hr.			4 hr.		
		Area	S.D.	Standard error of S.D.	Area	S.D.	Standard error of S.D.	Area	S.D.	Standard error of S.D.
		cm^2			cm^2			cm^2		
Adult males	174	3.04	.41	.02	6.22	1.00	.05	8.55	2.14	.11
Adult females	88	3.16	.48	.04	6.39	1.27	.10	8.40	1.82	.14
Estrogenic animals	94	2.98	.51	.04	5.27	1.04	.08	6.55	1.94	.14
Adult males with estrogens										
Experimental group before treatment	18	3.07	.40	.07	6.61	.99	.17	10.40	2.78	.47
Experimental group after treatment	18	2.76	.39	.06	5.02	1.25	.21	7.07	2.96	.50
Control group, beginning of experiment	18	2.83	.33	.06	6.51	.91	.15	9.72	2.74	.47
Control group, end of experiment	18	2.73	.33	.06	6.11	1.37	.23	10.36	3.59	.61
Adult females with estrogens										
Experimental group before treatment	17	3.24	.34	.06	6.21	.72	.12	9.24	1.45	.24
Experimental group after treatment	17	2.79	.40	.07	4.85	.56	.10	7.35	1.80	.31
Control group, beginning of experiment	17	3.09	.38	.07	6.49	.95	.17	9.68	2.20	.39
Control group, end of experiment	17	2.79	.33	.06	6.26	.92	.16	10.69	2.56	.45
Castrates with estrogens										
Experimental group before treatment	16	3.36	.45	.08	7.52	.97	.17	9.23	1.55	.26
Experimental group after treatment	16	2.89	.40	.07	5.41	.59	.11	5.83	1.02	.18
Control group, beginning of experiment	12	3.66	.61	.13	7.74	.70	.15	9.58	1.37	.29
Control group, end of of experiment	12	3.37	.34	.07	8.08	.98	.20	9.59	1.06	.23
Castrates										
Before castration	12	3.30	.77	.16	6.08	1.80	.38	7.98	1.83	.38
Smallest after castration	12	3.10	.37	.08	5.46	.94	.19	5.97	.94	.19
Stabilized after castration	11	3.74	.40	.08	7.35	.69	.14	9.30	4.00	.85
14 months after castration	4	3.00	.17	.06	7.54	.36	.14	9.15	.32	.12
Immature females	22	3.85	.55	.12	8.56	1.42	.31	12.58	2.38	.52
Pseudopregnant females										
Experimental group before treatment	9	3.26	.33	.08	6.57	1.16	.29	9.53	1.61	.40
Experimental group after treatment	9	2.94	.57	.14	5.26	.63	.16	7.10	.59	.15
Control group, beginning of experiment	5	3.34	.98	.34	6.73	.64	.23	8.84	2.06	.73
Control group, end of experiment	5	3.23	.63	.22	5.49	.96	.34	8.92	1.27	.45

Statistical methods. The usual method was employed. The small group method for "t" was used as described by Mainland (7). The probabilities (P) given in table 2 are taken from Fisher and Yates (8). A difference between 2 groups of data is not considered significant unless "P" is 0.01 or less.

Measurement of lesions. The technic is described in a preceding paper (4). The animals were measured at weekly intervals and oftener if marked variations occurred. The data given in table 1 have in every instance been checked by other measurements. Although measurements of the India ink spread were made immediately after injection and at 1, 2, 4, 24 and 48 hours, only the immediate, the 1 and 4-hour ones are included. The 24- and 48-hour measurements were difficult to make with any degree of precision and hence are omitted. The spread

TABLE 2. STATISTICAL COMPARISON OF THE SPREAD OF INDIA INK IN VARIOUS GROUPS OF ANIMALS

	Deg. freedom	4 hr.	
		t	P
Adult males with adult females	260	.54	0.6
Adult males with estrogens	266	7.50	0.000000001
Adult females with estrogens	180	6.61	0.000000001
Adult males with estrogens			
Control group, beginning of experiment with control group, end of experiment	34	.60	0.6
Control group, beginning of experiment with experimental group, beginning of experiment	34	.72	0.5
Control group, beginning of experiment with experimental group, end of experiment	34	2.79	0.01
Control group, end of experiment with experimental group, end of experiment	34	3.00	0.006
Control group, end of experiment with experimental group beginning of experiment	34	.03	0.97
Experimental group, beginning of experiment with experimental group, end of experiment	34	3.42	0.001
Castrates with estrogens			
Control group, beginning of experiment with control group, end of experiment	22	.01	0.9
Control group, beginning of experiment with experimental group, beginning of experiment	26	.63	0.5
Control group, beginning of experiment with experimental group, end of experiment	26	8.33	0.000000001
Control group, end of experiment with experimental group, end of experiment	26	9.46	0.000000001
Control group, end of experiment with experimental group, beginning of experiment	26	.71	0.4
Experimental group, beginning of experiment with experimental group, end of experiment	30	7.36	0.000000001
Castrates			
Before castration with smallest after castration	22	3.36	0.005
Before castration with largest after castration	21	1.87	0.1
Before castration with 14 months after castration	14	1.25	0.2
Largest after castration with 14 months after castration	13	.19	0.85
Largest after castration with smallest after castration	21	3.29	0.005
Adult females with immature females	37	2.36	0.02
Adult females with estrogens			
Control group, beginning of experiment with control group, end of experiment	32	1.22	0.2
Control group, beginning of experiment with experimental group, beginning of experiment	32	.71	0.4
Control group, beginning of experiment with experimental group, end of experiment	32	3.38	0.005
Control group, end of experiment with experimental group, end of experiment	32	4.39	0.0001
Control group, end of experiment with experimental group, beginning of experiment	32	2.07	0.05
Experimental group, beginning of experiment with experimental group, end of experiment	32	3.35	0.005
Pseudopregnant females			
Control group, beginning of experiment with control group, end of experiment	8	.06	0.95
Control group, beginning of experiment with experimental group, beginning of experiment	12	.72	0.4
Control group, beginning of experiment with experimental group, end of experiment	12	2.43	0.03
Control group, end of experimnet with experimental group, end of experiment	12	3.71	0.01
Control group, end of experiment with experimental group, beginning of experiment	12	.75	0.4
Experimental group, beginning of experiment with experimental group, end of experiment	16	5.19	0.000001

is usually at its height at 4 hours and all of our conclusions have been made from this measurement.

India ink. This was used in a dilution of 1 part India ink to 5 parts Locke's solution. An inoculum of 0.5 cc. was injected intradermally through a 25-gauge needle. Numerous experiments were also performed with an inoculum of 0.25 cc. on the smaller immature rabbits as their skin would not stand the larger amount. None of these, however, are included in the tables.

Hormone. The estrogenic hormone used was alpha-estradiol dipropionate.[3] This hormone was given in daily doses of 0.2 mg. in 1 cc. of sesame oil. The measurements listed in table 1 were made 3 weeks after beginning the injections. The male hormone was given 3 times a week for 3 weeks. The hormone used was testosterone propionate.[4] The dose used was 5 mg. in 1 cc. of sesame oil. All of the control animals received 1 cc. of sesame oil for the same period of time as the treated animals.

Anterior pituitary-like pregnancy urine extract[5] was used to produce pseudopregnancies. A total of 200 rat units was injected intravenously.

RESULTS

Variation in response. In order to show the marked variations which may occur in so-called normal rabbits, we have collected all of the data available on male rabbits,

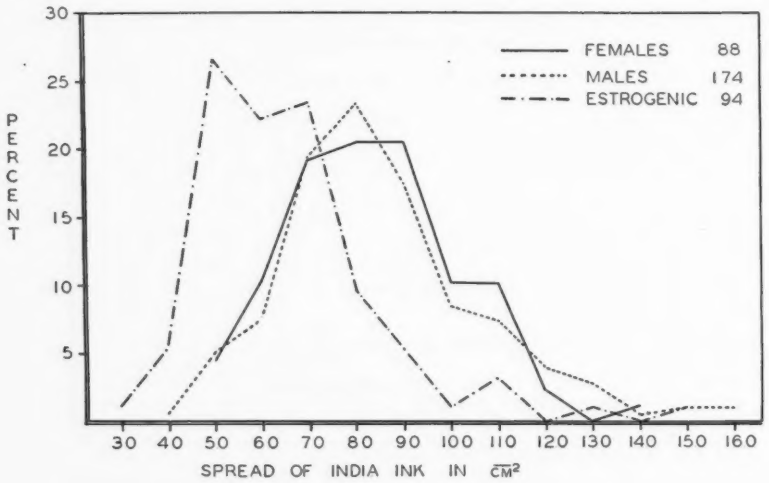

Fig. 1.

female rabbits which were not pseudopregnant, and all animals—male and female and castrate—which had received the estrogenic hormone. Also included with this last group are all the pseudopregnant females. Only a single measurement is listed from each male or female animal—the last measurement before treatment or before the animal was discarded. In some instances this was only a week after the rabbit was received. The estrogenic measurements are on rabbits which had received this

[3] The alpha-estradiol dipropionate (di-ovocylin) was kindly supplied by the Ciba Pharmaceutical Products Inc.

[4] The testosterone propionate was in part kindly supplied as perandren by the Ciba Pharmaceutical Products Inc., and in part as oreton by the Schering Corporation.

[5] The anterior pituitary-like pregnancy urine extract used was anturitrin-S. This was kindly supplied by Parke, Davis & Company.

hormone daily for 3 weeks, although a few are included which only received it for 10 days. The readings on the pseudopregnant animals were made 8 days after the injection of the anterior pituitary-like pregnancy urine extract. It is interesting to note that the curves as shown in figure 1 for males and females are almost identical and follow very closely the normal distribution curve. The curve for the estrogenic animals in addition to being moved to the left has also a definite skew in this direction. A statistical comparison of the data making up these 3 curves shows, as indicated in table 2, that the difference between the normal and the estrogenic is highly significant.

Castration. We were able to find no significant differences between the effect of castration on either immature or mature rabbits and none between males and females. In table 1 we have listed a small group of animals which have been followed over a long period of time. This group shows clearly that the effect of castration is at first to depress the spread of India ink and later to stabilize it at the original level. The depression lasts in some instances for as long as 6 months. This fact is important because castrated animals are frequently used by investigators only a month or so after castration as controls for male or female animals. As indicated by this data such practice is likely to cause grave errors.

Estrogenic hormone. This hormone has been tried in 4 groups of animals without producing significant differences. These 4 groups of animals were adult males, adult females, and castrates of both sexes at least 12 months after castration. Although the animals showed a similar depression in the India ink spread there were other differences which are worth reporting. The males receiving the hormone had a definite reduction in spermatogenesis. About half of the adult females showed a marked bacterial infection with necrosis of the uterus. The animals with this infection showed a reduction of the spread of India ink but did not change appreciably in their susceptibility to vaccinial infection. This is the only instance of divergence between these two methods which has so far been encountered and it is being investigated further. The female castrates were found to have a marked hypertrophy and hyperplasia of the uterus, with a small amount of infection and necrosis in a few instances. The male castrates showed no gross changes.

Pseudopregnancy. The effect of pseudopregnancy has been previously reported (4). But in order to check these results an experiment similar to the one with the estrogenic hormone as shown in table 1 was performed. The results were similar to those obtained with the estrogenic hormone.

Immature rabbits. A large number of studies on immature rabbits have been made. They include observations on a few rabbits while still nursing and on over 100 which were obtained from a dealer just after weaning. These results have been difficult to interpret, since in all but one group of females, which is given on the chart, extensive weekly variations are evident. It was impossible to get any data on the nursing rabbits. The last group of animals studied shows a consistent rise of India ink for 4 weeks, followed by stabilization at a relatively high figure. We are inclined to think that this rise is no more than the immediate rise we find in all rabbits which are obtained from dealers. Table 2 shows this measurement not to be significantly different from a group of normal adult females. Further study is needed of the effect of age both on the spread of India ink and on susceptibility to infection before any definite conclusions can be drawn.

Male hormone and spermatogenesis. Our findings now indicate that testosterone propionate given to immature male and female rabbits has no effect on the spread of India ink in the skin. Susceptibility to infection is similarly unchanged. Spermatogenesis, as pointed out above, does seem to be related to the spread of India ink and

the resistance of the rabbit to infection. We have as yet no quantitative experiments on this subject, but we hope to report such experiments in the near future.

DISCUSSION

Since the distribution of bacteria and viruses has been shown to parallel the spread of India ink, the facts brought out in this paper make possible a better understanding of numerous diseases which are known to be associated with various endocrine states. While we do not wish to imply that concentration or dispersion of the disease-incitant in the skin or elsewhere is the only factor affecting disease processes we do believe that it is an important one.

Duran-Reynals (1) pointed out several years ago that dispersion of bacteria in the skin will enhance the lesion produced if either the concentration of the bacteria is so high that dispersion fails to reduce it below the critical level or the virulence of the bacteria is so great that the local agencies are insufficient to destroy them even when widely separated in the lesion.

In a previous experiment (5) we showed that when the vaccine virus was localized by the estrogenic hormone or pseudopregnancy a larger amount was neutralized than if allowed to spread over a larger area.

Further work is needed on the effect of dispersion and concentration of both bacteria and viruses. But the information now available indicates that certain disease agents such as vaccinia are aided by anything that causes an increased spread in the skin and others, such as moderately virulent staphylococci, are inhibited by dispersion. The converse is also true.

SUMMARY

Experiments are described which show that wide variation in the spread of particulate matter in the skin occurs in so-called normal rabbits. However, under controlled conditions this spread can be reduced.

The estrogenic hormone and pseudopregnancy significantly reduce this spread.

The effect of castration is reduction of this spread which later returns to normal though this latter may take as long as 6 months.

A few experiments indicate that the male hormone does not affect the spread of India ink but that the amount of spermatogenesis present in the adult male is closely related to the susceptibility of the animal to infection.

The relation of this spread to susceptibility to infection is shown to be close.

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THE EFFECT OF A NON-ANDROGENIC TESTIS EXTRACT ON THE ESTROUS CYCLE IN RATS

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IT HAS BEEN DEMONSTRATED that one characteristic of the androgens is that of depression of the gonadotropic activity of the anterior lobe of the pituitary. This subject has been studied in this laboratory and reviewed by Cutuly and Cutuly (1). It is thought that it is because of this characteristic that androgens in adequate dosages will depress the estrogenic cycle in the female rat. It also has been known that lipoid-insoluble non-androgenic extracts of the testes could be obtained which would cause atrophy of the secondary sex organs of the male rat. The most recent and most striking demonstration of this effect is due to Vidgoff (2). The present publication deals with the effect of non-androgenic testicular extracts on the estrous cycle of the female rat.

Unpublished results by McCullagh, Deeton and Cutuly[1] indicated that whole beef testicular substance to the extent of 15% of the daily diet definitely depressed the sex cycle of the female rat. The average length of the cycle of the group studied at that time was 4.6 days. The diet of beef testes was continued for a period of 32 days. During this time the average length of the cycle was increased to 8.1 days. Most of the rats rapidly returned to normal when the beef testes substance was omitted from the diet. The animals did not lose weight and there were no other signs of dietary deficiency.

Recently, we decided to extend these experiments in order to determine whether or not the concentrated preparations of inhibin (the name employed for the water-soluble hormone, 3) which are now available would cause more striking results.

THE PREPARATION OF SOLUTIONS OF INHIBIN

The first experiments were performed by using glandular extracts prepared according to the instructions of Vidgoff (2) who used the method of Deutsch, Eggleton and Eggleton (4) to obtain a protein-free aqueous concentrate of the glandular material. He removed all fat-soluble material from the extracts by washing them with benzene, thus extracting any free steroids that might be present. Preparations made according to these instructions proved more active in depressing the sex cycle of the female rat than anything we had employed before this time. However, we encountered certain technical difficulties and it seemed desirable to alter the method somewhat in order to prepare large quantities of the extract for experimental purposes.

The method which we employ at present for preparing crude extracts of bovine testes is as follows. Freshly removed glands are frozen at the abbatoir and are kept frozen until used. The glandular substance is ground with a meat grinder to a fine mush and then rapidly heated to 65° C. by passing superheated steam into the mush.

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[1] Reported to the Cleveland Section of the Society for Experimental Biology and Medicine in January, 1935.

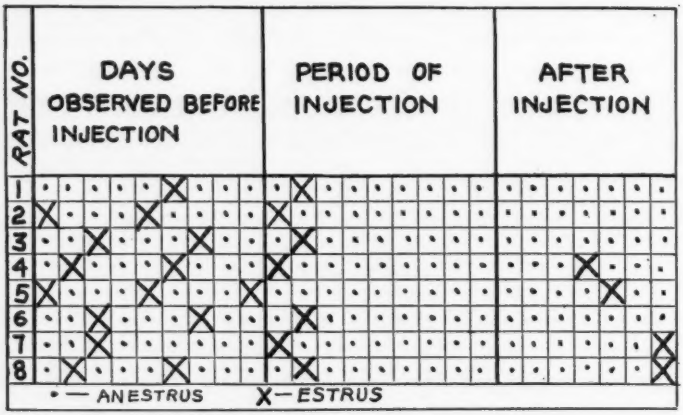

Fig. 1. The effect of 9 successive injections of 1 cc. of inhibin extract on estrous cycle of rat.

Vigorous stirring is continued throughout the heating process. As a result of this treatment, a considerable portion of the proteins are precipitated. These can be removed rapidly, first by expressing the mush through cheesecloth, and then by forcing the expressate through a Sharples centrifuge, using a large syringe as a pump. The yield at this point should be 600 to 700 cc. from each kg. of glandular material used. Forty grams of anhydrous sodium sulfate is added to each 100 cc. of solution and the mixture is stirred at a temperature of approximately 32° C. until the salt is in solution. The precipitated proteins are removed by filtration through flannel cloth. The resulting clear filtrate is then cooled to 0° C., stirring continuously so that only small crystals form. These crystals are then removed by filtration through cloth at a temperature of 0° to 4° C. About 250 to 300 cc. of filtrate is obtained from each kg. of gland tissue used. The filtrate is extracted with dibutyl ether to remove free steroids. The aqueous extract is then evaporated either in open pans with a fan or in vacuum to such a volume that 1 cc. represents 25 gm. of fresh tissue.

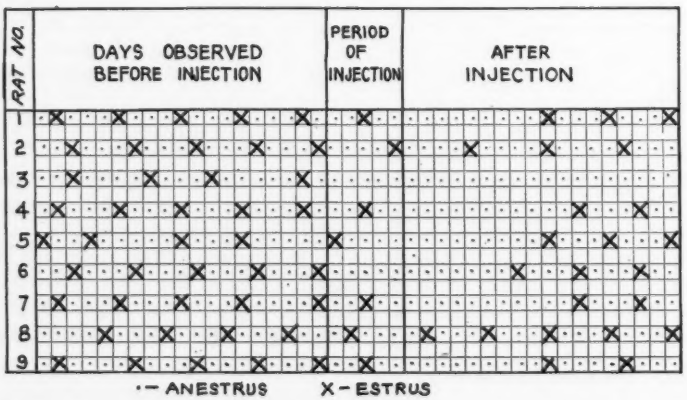

Fig. 2. The effect of 5 successive injections of 1 cc. of inhibin extract on the estrous cycle of the rat.

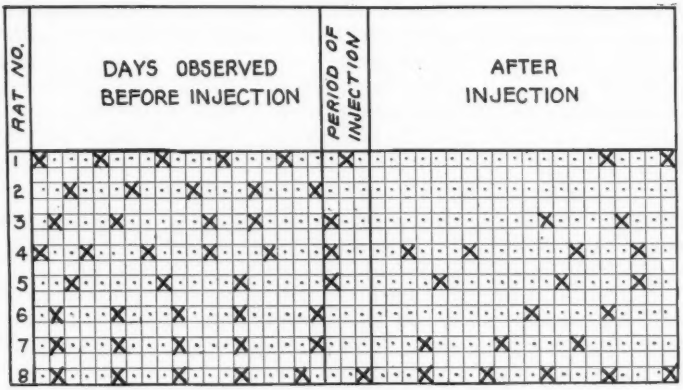

Fig. 3. The effect of 3 successive injections of 1 cc. of inhibin extract on the estrous cycle of the rat.

THE INTERRUPTION OF THE ESTROUS CYCLE IN THE FEMALE RAT AS THE RESULT OF THE INJECTION OF INHIBIN

It has been found that extracts of testes prepared according to the above method are very effective in stopping the estrous cycle of normal mature rats. Figures 1 to 3 demonstrate this phenomenon. The group listed in figure 1 was injected with 1 cc. of extract daily for 9 days after it had been established that the rats were normal in regard to their sexual cycles; the rats listed in figure 2 were injected with 1 cc. daily for 5 days; and those in figure 3, with 1 cc. daily for 3 days. Figure 4 records results on a control group of rats which received 1 cc. of liver extract for 5 days. The liver extract was prepared by the same technic as was employed in the preparation of the testicular material.

The crosses in the tables indicate that the rat in question showed a positive estrous smear on that day. The perpendicular lines segregate the days in the various

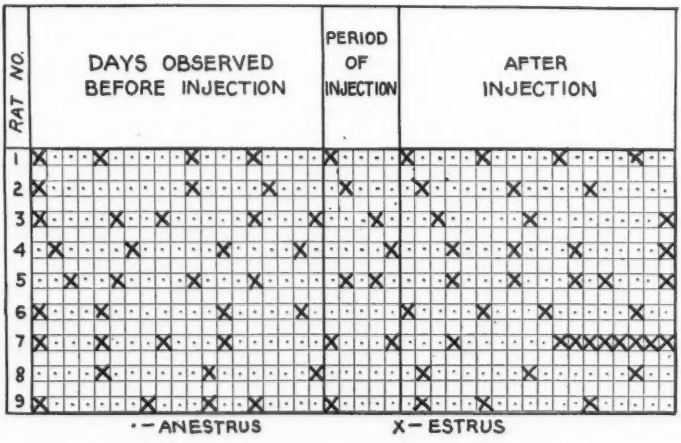

Fig. 4. The effect of 5 successive injections of liver extract.

experiments during which the animals were being observed. It will be noted that in all the experiments, only rats which had been demonstrated to have normal cycles were used. As seen in figure 1 9 consecutive injections caused a complete cessation of estrus until some days later. In fact, some of the animals had not returned to normal when the experiment was terminated a week after the final injection. Treatment with the extract for a period of 5 days (fig. 2) caused a marked disturbance in the estrous cycle of 7 of 9 rats. These 7 animals did not recover for more than a week after receiving the extract. When the animals were injected for 3 days (fig. 3) only 4 of 8 animals were seriously disturbed in regard to the regularity of the cycle. This is significant, however, when one considers that in the control group injected with liver (fig. 4) there was no disturbance in the cycles.

These experiments have been repeated several times and have been adequately confirmed. A subsequent publication will report experiments which demonstrate the sensitivity of female rats to inhibin at various stages of the estrous cycle. Also, a method for the assay of inhibin based on these findings will be described.

SUMMARY

Non-androgenic testicular extracts (inhibin) will cause cessation or interruption of the estrous cycle of the normal female rat.

The authors are indebted to Mr. Owen Reeves and Mr. Wendell Bowman for technical assistance in the early part of this work.

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THE EFFECT OF DIETHYL STILBESTROL DIPROPRIONATE ON ENDOMETRIAL TRANSPLANTS

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IN THE COURSE of experimental work on the possible toxic effect of the powerful synthetic estrogen stilbestrol, introduced by Dodds and coworkers (1), we constantly observed marked enlargement and congestion of the uterus and uterine tubes. This enlargement resulted from the estrogenic 'priming,' and the question arose as to whether or not endometrium obtained from these 'primed' animals could be successfully transplanted and if so, what would be its fate under continued administration of estrogenic substance. The possible outcome interested us in the light of the advocacy of the procedure of preserving a portion of the endometrium after supravaginal hysterectomy (2–11), especially when it is remembered that these patients frequently are treated later for hypoestrinism syndromes, and in consideration of the danger of carcinogenic effect of the synthetic estrogens discussed in many quarters (12–16).

Transplantation of the endometrium has been successfully carried out many times since the early attempts of Goldzieher (11) and the more extensive ones of Stilling (17, 18). Experimental efforts were increased after Sampson's theory of endometriosis was expounded (19, 20–23), and the work of Jacobsen (24, 25, 26) and many others soon followed. Recently Harbitz (27) has extensively reviewed the literature in this field, and Hagino (28–31) and others have undertaken the study of hormonal influences on transplants. Our own interests were confined to the questions: (*a*) Can estrogenically 'primed" endometrium be successfully transplanted? (*b*) What would be the fate of such transplants under the influence of continued estrogenic administration with the synthetic estrogen, diethyl stilbestrol diproprionate?

MATERIAL

The experimental animals were adult virgin chinchilla rabbits, weighing approximately 3 lb. All animals were healthy and were maintained in segregation under optimum conditions. For the purposes of this study, they were divided into 2 groups consisting of 7 animals each. In each group, 2 animals were used as controls, the others as experimental animals.

To the experimental animals of group 1 the diproprionate ester of diethyl stilbestrol (32) was administered intramuscularly in daily doses of 12,500 I.U. until 600,000 I.U. had been given. At this time all animals of the group, experimental as well as controls, were laparotomized under general anesthesia, using aseptic methods. The effect of diethyl stilbestrol diproprionate on the reproductive tract of the experi-

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mental animals was fairly marked. It consisted of extreme hypertrophy of the uterine cornua, with marked vascular engorgement, and punctate to fairly large scattered areas of necrosis, probably ischemic in origin. The oviducts and ovaries showed no change, though the vagina was hypertrophied. A section of the uterine cornu, about 1 cm. in length near the central part, was isolated between ligatures and removed, the raw ends being approximated. The removed section was incised in its long axis and laid open. Squares were cut through the entire uterine wall, measuring 3 to 4 mm. across. These tissues were transplanted to new sites in the peritoneal cavity. In each instance the transplant was sutured to its new site by interrupted black silk sutures, with the serosal surfaces in apposition. Black silk was used to facilitate identification of the transplants at a later date. The uterine tissue was attached to the following sites: (*a*) to the mesenteric border of the small intestine; (*b*) to the peritoneum of the anterior abdominal wall, 3 cm. to the left of the midline and 4 cm. cephalad the symphysis; (*c*) to a corresponding site on the right of the midline. The same procedure was followed in each of the control animals of this group.

Injections of diethyl stilbestrol diproprionate were continued in the experimental animals for 30 days, an additional dosage of 375,000 I.U. Laparotomy was then performed to determine the fate of the transplanted tissue. Since large cystic masses were found at the sites of transplantation, the animals were killed, and the tumors were removed for histological study. In no instance was transplantation unsuccessful. Grossly, the transplants situated on the abdominal wall appeared as translucent cystic masses, ranging in diameter from 4 to 7 cm., and containing a yellow-tinged serous fluid. There were no grossly evident abnormalities in the adjacent abdominal walls, and the transplants showed no areas of necrosis.

In the control animals of this group, transplantation was also successful. In contrast to the large cystic masses found in the experimental animals, the transplants in the control animals were extremely small, not grossly cystic, and they showed less vascular engorgement. In these also no gross abnormalities were found in the surrounding tissues.

The experimental animals of group 2, as in group 1, received 600,000 I.U. of diethyl stilbestrol diproprionate prior to laparotomy. The experimental procedure previously described was followed in this group. Postoperatively, the drug was administered daily to the experimental animals until an additional 525,000 I.U. was given. Laparotomy was then performed for inspection of the transplants. The animals were killed, and the transplanted tissues were removed for study. No failures of transplantation occurred.

On gross examination, the transplants were structurally similar to those of group 1, with the exceptions (*a*) that the cystic masses were larger in group 2, and (*b*) that several showed gross hemorrhage into the cysts. In this group as in group 1 the control animals showed small poorly developed transplants.

Microscopical Examination

Group 1. Experimental. The microscopical sections of all the transplants showed viable endometrial tissue, including the glandular elements and tunica propria, as well as the myometrium. The myometrium could be identified, but showed no cellular activity. In some instances fibrotic changes could be seen. The area of greatest growth was that of the endometrium. The glandular elements varied considerably in different sections. In each section there were large cystic spaces lined with simple epithelium. This epithelium varied from low cuboidal to high columnar, depending upon the size of the cyst, in inverse proportion. The cells were adult in type and no mitotic figures or invasive characteristics could be detected. Smaller cysts were also

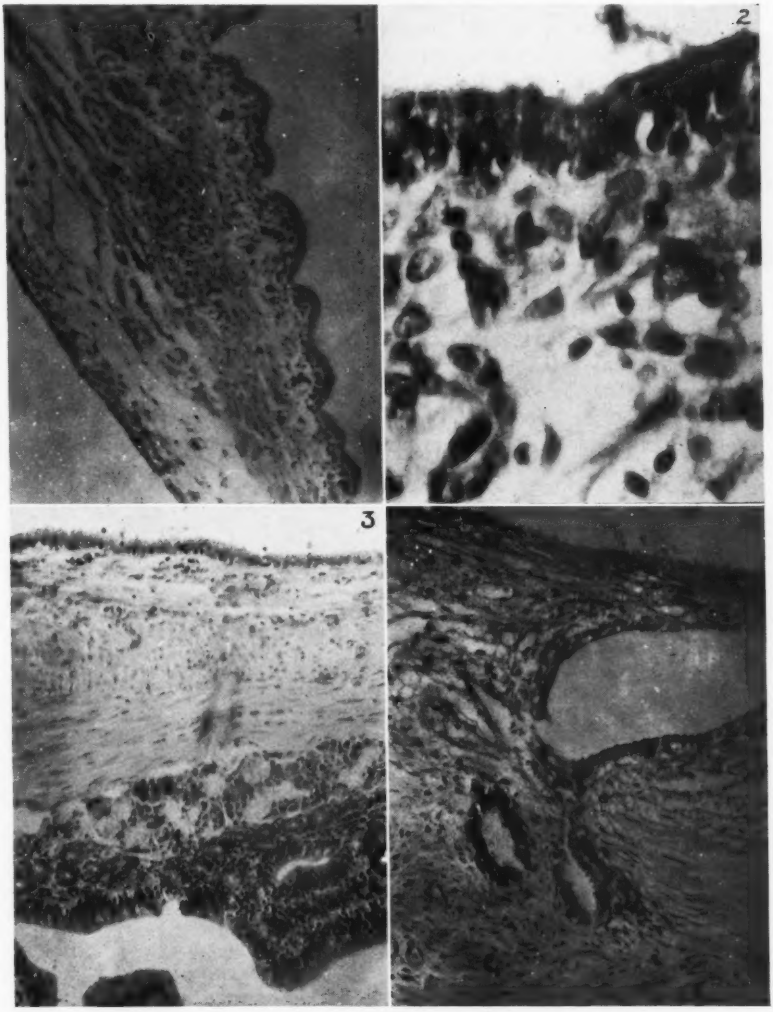

Fig. 1. EXPERIMENTAL ANIMAL OF GROUP 1. Section shows endometrial wall of 2 adjacent cysts. Both cysts are lined with low columnar epithelium. The loose fibrous stroma shows numerous capillaries. Fig. 2. SECTION OF TRANSPLANT OF EXPERIMENTAL ANIMAL GROUP 1. High power, showing tall columnar epithelium and loose fibrous stroma of tunica propria. Fig. 3. CONTROL ANIMAL OF GROUP 1. Section shows both cyst of transplant and wall of small intestine. Here the endometrium is moderately tall columnar. The stroma is compact and capillaries are infrequently distinguished. Fig. 4. EXPERIMENTAL ANIMAL OF GROUP 2. Section of transplant cyst wall, showing gland formation. Endometrium of cyst wall is low columnar, whereas that of glands is tall columnar. The stroma is slightly compressed, yet is not dense. Dilated capillaries are numerous.

found near the attachment of the large cysts to the site of implantation. Here the lining epithelium was almost always tall columnar in type. The nuclei of these cells tended to be ovoid, vesicular, and basal, although areas of pseudostratification could

also be seen. The cytoplasm of these cells was granular, and usually devoid of vacuoles. No secretory substance could be seen in the lumina of these glands. The tunica propria about these cysts consisted of loose fibrous stroma, with congested vessels and moderate edema (fig. 1, 2). No infiltration of plasma cells or round cells was evident in the transplanted tissue, with the exception that infiltration was marked about the silk sutures. The only change demonstrable in the host tissue was vasodilation, of moderate extent in the vessels of the intestine, and much less evident in the abdominal wall.

The control sections of group 1 showed similar microscopic structure (fig. 3). Here, however, all the cysts were small and consistently lined with simple low columnar epithelium. No evidence of increased cellular activity could be seen. The tunica propria was more compact, and no vascular engorgement was in evidence.

Group 2. Experimental. In the experimental animals of group 2, the cysts were larger in size, and less often, smaller cysts were found in conjunction. These cysts, in almost every instance, were lined with simple low cuboidal epithelium. In some areas both low cuboidal and moderately high columnar epithelium were found. A few cysts showed spur formation, but papillary ingrowths were not demonstrable. The cells of the epithelium were healthy, with round, basal nuclei and granular cytoplasm. Vacuolization of the cytoplasm was absent, and no mitosis could be found. The tunica propria in this group was compressed, but no increase in fibrosis was noticeable (fig. 4). Vascular engorgement was again a prominent feature in these sections, and several of the larger cysts were filled with hemorrhagic material, showing organization. As in the sections of group 1, the myometrium could be readily identified in the transplants, and the host tissue showed only increased vascularity.

The control animals of this group showed no variation from those of group 1.

DISCUSSION

Endometrium has been successfully transplanted by many investigators (27), indicating its value as an experimental means of corroborating clinical findings. The percentage of successful transplants in many of the previous experiments has been low. We feel that the uniformity of successful transplants in our experimental animals was achieved by the utilization of stilbestrol as a stimulant to endometrial growth. Despite the marked proliferation of the transplanted endometrium and extensive cystic change, there was no evidence of secondary transplants or of extrusion of endometrium through the cyst walls. The marked hyperemia observed grossly and confirmed on microscopic study is probably to be attributed to stilbestrol, since it was absent in the control animals. Hyperemia has been demonstrated as an occurrence incident to estrogenic administration (33). In our opinion this hyperemia may largely account for the success of the transplants and the rapidity of their development. The absence of adhesions to the transplants (cysts) is noteworthy, and is in contrast to the usual pathological findings in clinical endometriosis. It can of course be explained by the fact that no rupture of the cysts or extrusion of endometrium occurred. The absence of cyclic changes and the infrequency of hemorrhage into the cysts may account for the failure of these cysts to rupture with subsequent secondary seeding.

The stimulating effect of the estrogen, diethyl stilbestrol diproprionate, on endometrial transplants, leads us to caution against the use of massive dosage of estrogens postoperatively in women castrated to decrease symptoms of endometriosis, who are later treated for hypoestrinism syndromes. In these patients therapy may stimulate further development of the endometrial tissue present, and cause recurrence of symptoms. This fact should be emphasized in view of our clinical experience that patients

with surgical menopausal syndromes require large doses of estrogen to alleviate their symptoms (34, 35, 36).

The utilization of diethyl stilbestrol diproprionate may be of value in the further study of heteroplastic endometrium, particularly when there is a desire to secure adequate transplants for the observation of the effect of drugs on the growth and development of this tissue.

It is interesting, in view of the widely discussed carcinogenic activity of the powerful estrogens that we observed no invasive tendency nor increased cellular activity in these growths.

SUMMARY

Uniformly successful transplantations of endometrium were accomplished in 10 adult female chinchilla rabbits utilizing mesenteric and peritoneal transplants of estrogenically (diethyl stilbestrol diproprionate) 'primed' endometrium.

Under the influence of massive dosage of diethyl stilbestrol diproprionate (975,000 I.U. and 1,125,000 I.U.) these transplants developed into large cystic masses, which contained viable endometrium. No tendency toward invasive or increased cellular activity was evidenced in these transplants.

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CREATINE-CREATININE METABOLISM AND THE HORMONES

VI. THE EFFECT OF PARENTERAL INJECTION OF PITRESSIN, WITH AND WITHOUT CREATININE, PITOCIN, DESICCATED THYROID, PARATHYROID EXTRACT, INSULIN AND ZINC INSULIN, UPON MUSCLE CREATINE AND CREATININE-CREATINE EXCRETION IN THE RAT TOGETHER WITH OBSERVATIONS UPON THE CREATININE-CREATINE TRANSFORMATION UNDER THE INFLUENCE OF THE POSTERIOR PITUITARY HORMONES

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WE HAVE SHOWN in several studies from this laboratory that there is a close relationship between the metabolism of the amino acids and related substances, water and salt with that of creatine and creatinine (1–4). From these results it is to be expected that those hormones, such as the adrenal cortical hormone (5) and pitressin, which play a part in salt and water metabolism in the body, should likewise affect the metabolism of creatine and creatinine. In this paper the effects of injection of the different hormone preparations listed below upon creatine-creatinine metabolism will be reported.

EXPERIMENTAL

The experimental technic used has been described in the above publications from this laboratory (1–4). The preparations used and the amount of fluid injected were as follows.

Hormone	Units per cc.	Amount of fluid injected, cc.
Pitressin	10 pressor, 0.4 oxytocic	0.1 to 0.6
Pitocin	10 oxytocic, 0.4 pressor	0.1 to 0.4
Desiccated thyroid	1 mg.	0.1 to 0.4
Parathyroid	100	0.8 to 1.6
Zinc insulin	4	0.2 to 0.8
Insulin	10	0.1 to 0.2

The amount of fluid injected must be known in order to correctly interpret the results of the creatinuria. Injections of less than 1 cc. of water had only a small effect upon the creatinuria in previous studies. The creatinuria after parathyroid injection was considered to be due to the fluid injected. That obtained after Zn insulin was too great to be due to the fluid injected. The fluid medium was considered of no effect upon the creatinuria observed in the rest of the cases. In several studies the color of the Jaffe reaction was destroyed by the creatinine enzyme. None of the hormones injected gave this reaction when mixed with alkaline picrate. Our results are based upon the assumption that the creatine-creatinine excretion without the hormones would remain constant from day to day.

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Pitressin. The injection of 1, 2, 3 and 4 U of this hormone caused total increases in extra creatine excretion of 22, 30, 82 and 150 mg., respectively (fig. 1, 2). A slight retention of creatinine occurred on the first 3 doses and on 4 U, 36 mg. were retained. This last result will assume some significance as the following discussion will show. Since the increases in creatine excretion were much larger than the creatinine retentions it is certain that the hormones, in these particular experiments, with 2 exceptions, stimulated creatine excretion.

Creatinine. The injection of 10, 20, 30 and 40 mg. of creatinine caused increases of creatine excretion of 105, 155, 160 and 120 mg., respectively, accompanied by increases in creatinine excretion of 30, 35, 47 and 26 mg., respectively (fig. 1, 2). These results confirm those published several times from this laboratory, namely, that in-

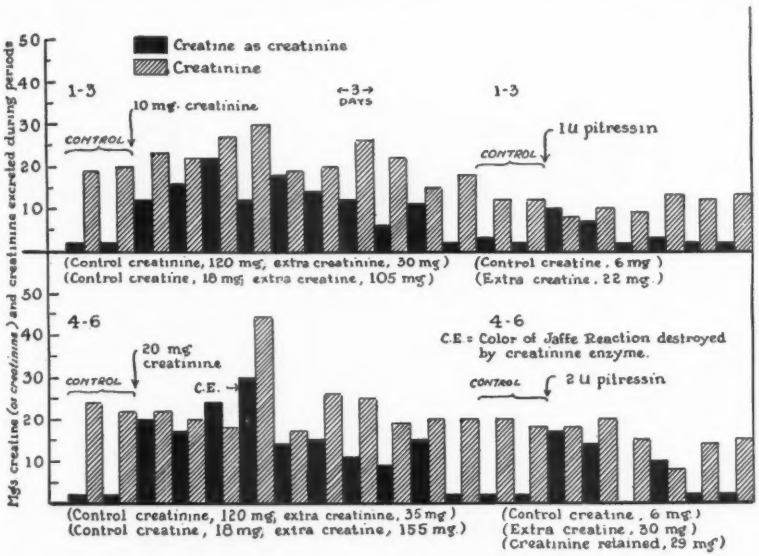

Fig. 1.

jected creatinine stimulates the excretion of both creatine and creatinine in the urine (4).

Pitressin and creatinine. When 10, 20, 30 and 40 mg. of creatinine were injected separately, but at the same time with 1, 2, 3 and 4 U of pitressin, the retention of creatinine formed from protein metabolism was 41, 48, 0 and 50 mg., respectively, accompanied by an increased excretion of creatine of 33, 35, 50 and 46 mg., respectively (fig. 3). After injection of 10 to 40 mg. of creatinine alone (fig. 1, 2) a total of 138 mg. of extra creatinine excretion was obtained compared to a retention of 139 mg. of body creatinine after the injection of the same doses of creatinine with 1 to 4 U of pitressin. Since the injected creatinine was excreted as such it is evident tha tthis hormone causes a retention of the creatinine formed from protein metabolism and has no action on injected creatinine.

It was also observed that, after the creatinine injections, a retention of creatinine for 9 to 12 days occurred. Later, however, more creatinine was excreted than was injected (fig. 1, 2) accompanied by large increases in creatine excretion.

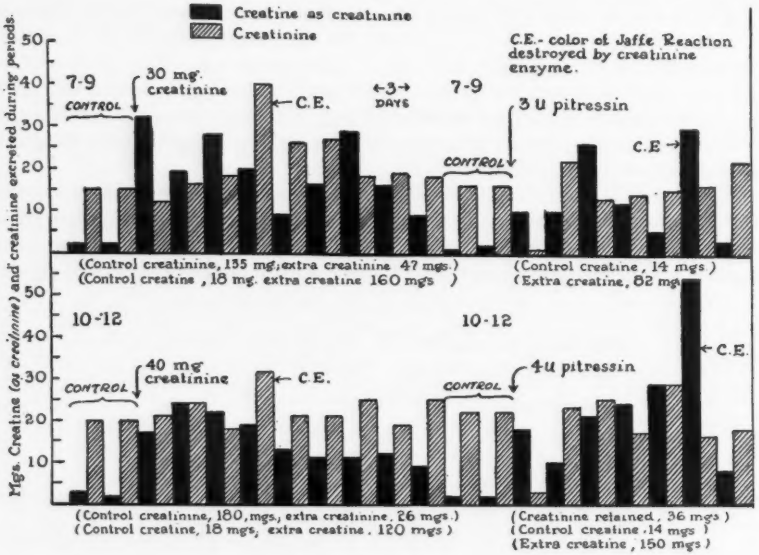
Creatine as creatinine
Creatinine
C.E.- color of Jaffe Reaction destroyed by creatinine enzyme.
Mgs. Creatine (as creatinine) and creatinine excreted during periods.
7-9
30 mg. creatinine
CONTROL
C.E.
←3→ DAYS
7-9
3 u pitressin
CONTROL
C.E.
(Control creatinine, 135 mg; extra creatinine 47 mgs.)
(Control creatine, 18 mg. extra creatine 160 mg's)
(Control creatine, 14 mgs)
(Extra creatine, 82 mgs)
10-12
40 mg creatinine
CONTROL
C.E.
10-12
4u pitressin
CONTROL
C.E.
(Control creatinine, 180, mgs, extra creatinine, 26 mgs.)
(Control creatine, 18 mgs, extra creatine, 120 mgs)
(Creatinine retained, 36 mgs)
(Control creatine, 14 mgs)
(Extra creatine, 150 mgs)

Fig. 2.

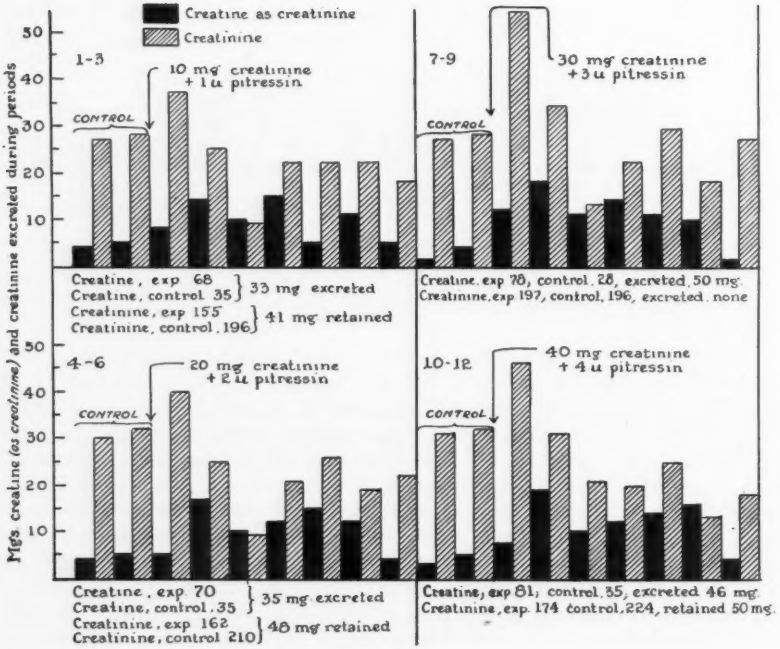
Creatine as creatinine
Creatinine
Mgs. creatine (as creatinine) and creatinine excreted during periods.
1-3
10 mg creatinine + 1 u pitressin
CONTROL
7-9
30 mg creatinine + 3 u pitressin
CONTROL
Creatine, exp 68
Creatine, control 35
33 mg excreted
Creatinine, exp 155
Creatinine, control, 196
41 mg retained
Creatine, exp 78, control, 28, excreted, 50 mg.
Creatinine, exp 197, control, 196, excreted, none
4-6
20 mg creatinine + 2 u pitressin
CONTROL
10-12
40 mg creatinine + 4 u pitressin
CONTROL
Creatine, exp 70
Creatine, control, 35
35 mg excreted
Creatinine, exp 162
Creatinine, control 210
48 mg retained
Creatine, exp 81, control, 35, excreted 46 mg.
Creatinine, exp. 174 control, 224, retained 50 mg.

Fig. 3.

Pitressin. In view of the above results this hormone was again injected in similar doses into the same animals as studied above. The results obtained are given in figure 4. For the first 3 or 4 experimental periods (9 to 12 days) after the injection of the hormone there is a retention of creatinine accompanied by a corresponding increase in creatine excretion. The creatinine excretion then rises from 12 to 18 days, falls again at the 21st day, then begins a final rise again to the normal value from 21 to 27 days.

The values listed in table 1 bring out further the effect of pitressin on the creatinine→creatine transformation in the rat. In 7 experiments (fig. 1, 3, 4) where 1 to

TABLE 1. EFFECT OF PARENTERAL INJECTION OF PITRESSIN AND PITOCIN UPON THE CREATININE-CREATINE TRANSFORMATION AND EXCRETION IN THE BODY

Hormone	Units	No. of rats	Exp. days	Creatinine			Creatine (as creatinine)		
				Exp.	Control	Retained	Exp.	Control	Excreted
				mg.	mg.	mg.	mg.	mg.	mg.
Pitressin	2	3	18	120	149	29	36	6	30
Pitressin	1[1]	3	21	155	196	41	68	35	33
Pitressin	2[1]	3	21	162	210	48	70	35	35
Pitressin	4[1]	3	21	174	224	50	81	35	46
Pitressin	2	3	24	211	240	29	118	80	38
Pitressin	3	3	27	205	270	65	126	54	72
Pitressin	6	3	24	186	248	62	137	64	73
				Total		324			327
				Average		46			47
Pitocin	1	3	24	143	210	67	65	14	51
Pitocin	2	3	27	112	175	63	73	21	52
Pitocin	3	3	24	171	240	69	116	32	84
Pitocin	4	3	27	192	216	24	112	45	67
				Total		223			254
				Average		56			64
Pitocin	2	3	27	220	189	31[2]	115	36	79
Pitressin	1	3	18	74	72	0	28	6	22
Pitressin	3	3	21	110	119	9	96	14	82
Pitressin	4	3	21	190	154	36	164	14	150
Pitressin	3[1]	3	27	197	196	0	78	28	50

[1] In these experiments creatinine was also injected. Since this creatinine was promptly excreted the values here also represent the retention of creatinine formed from the usual protein metabolism in the body.

[2] This 31 mg. of creatinine was excreted and not retained.

6 u of the hormone were injected, the total retention of body creatinine was 324 mg. accompanied by an increase creatine (as creatinine) excretion of 327 mg. (or 327 × 1.16 = 379 mg. of creatine as such). We conclude that this retained creatinine was quantitatively transformed into creatine, the excess of which was then excreted into the urine. This agreement is all the more remarkable when it is considered that pitressin, as well as pitocin, in the doses injected, both increased the muscle creatine of these rats from 10 to 17% above normal (table 2). Our control muscle creatine values are based on the average muscle creatine of 552 animals used in previous studies and comparable in every way to those used as controls in the present study. The above values show that the extra creatine excretion observed in the present studies was not obtained from the muscle creatine.

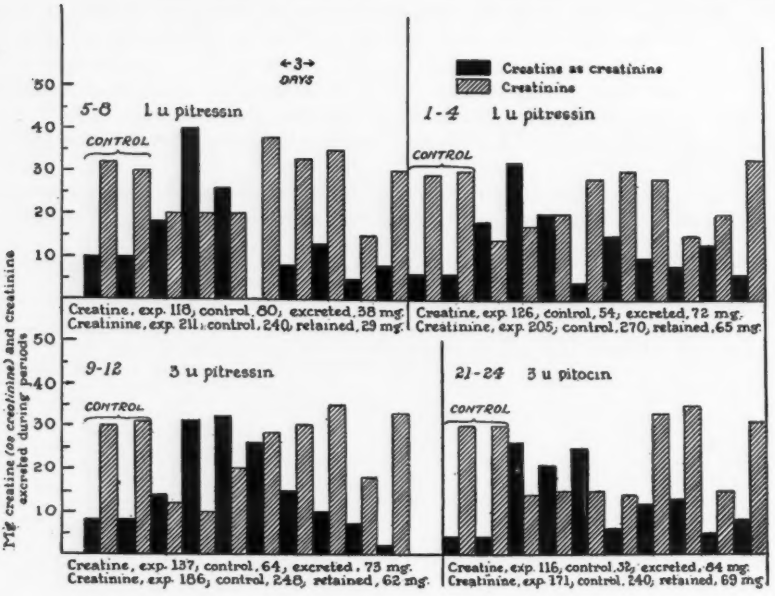
Creatine as creatinine
Creatinine
←3→ days
5-8 1 u pitressin
1-4 1 u pitressin
CONTROL
Creatine, exp. 116, control, 80, excreted, 38 mg.
Creatinine, exp. 211, control, 240, retained, 29 mg.
Creatine, exp. 126, control, 54, excreted, 72 mg.
Creatinine, exp. 205, control, 270, retained, 65 mg.
9-12 3 u pitressin
21-24 3 u pitocin
Creatine, exp. 137, control, 64, excreted, 73 mg.
Creatinine, exp. 186, control, 248, retained, 62 mg.
Creatine, exp. 116, control, 32, excreted, 84 mg.
Creatinine, exp. 171, control, 240, retained, 69 mg.
Mgs creatine (as creatinine) and creatinine excreted during periods

Fig. 4.

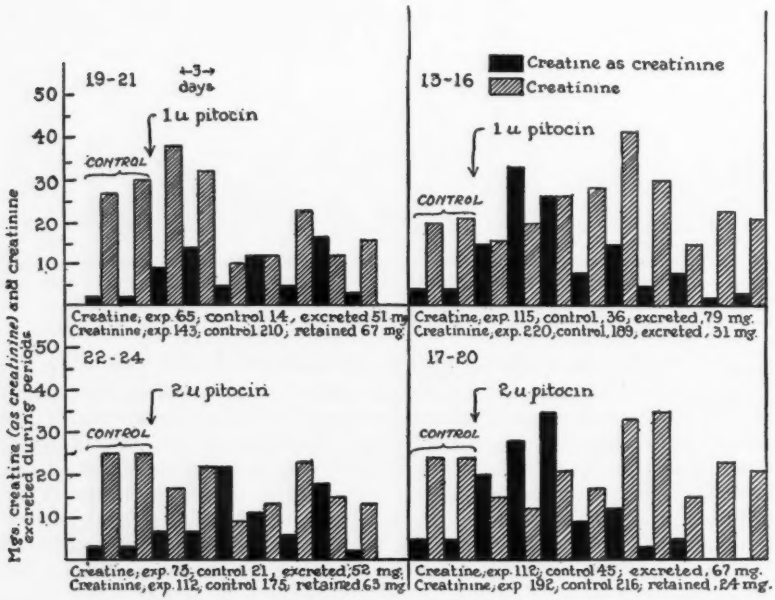
Creatine as creatinine
Creatinine
19-21 ←3→ days
1 u pitocin
13-16 1 u pitocin
CONTROL
Creatine, exp. 65, control 14, excreted 51 mg.
Creatinine, exp. 143, control 210, retained 67 mg.
Creatine, exp. 115, control, 36, excreted, 79 mg.
Creatinine, exp. 220, control, 189, excreted, 31 mg.
22-24 2 u pitocin
17-20 2 u pitocin
Creatine, exp. 75, control 21, excreted, 52 mg.
Creatinine, exp. 112, control 175, retained 63 mg.
Creatine, exp. 112, control 45, excreted, 67 mg.
Creatinine, exp. 192, control 216, retained, 24 mg.
Mgs creatine (as creatinine) and creatinine excreted during periods

Fig. 5.

Pitocin. Practically the same results were obtained with this hormone (fig. 4, 5; table 1, 2). In 4 experiments the injection of 1 to 4 U caused a total retention of 223 mg. of creatinine which was accompanied by an increased creatine, as creatinine, excretion of 254 mg.

A somewhat different story is revealed in the results of another 5 studies, 1 with pitressin and 4 with pitocin, shown at the bottom of table 1. Creatinine retention, however, except in 1 case, was not obtained in these studies, while in every case an increased creatine excretion occurred. In these studies it is also evident that these

TABLE 2. EFFECT OF PARENTERAL INJECTION OF VARIOUS HORMONES UPON MUSCLE CREATINE

Hormone	Amount injected	Exp.	No. of animals	Muscle Creatine			Increase over controls
				Max.	Min.	Ave.	
		days		%	%	%	%
Controls			43	0.45	0.38	0.42	
Insulin	1–2 U	1	4	0.44	0.40	0.43	None
Zink insulin	1.8 U	1	4	0.40	0.39	0.40	None
Desiccated thyroid	0.2 mg.	1	4	0.44	0.41	0.43	None
Parathyroid	8.3 U	1	2	0.40	0.39	0.40	None
Parathyroid	16.6 U	1	2	0.41	0.39	0.40	None
Pitressin	1 U	1	2	0.49	0.46	0.47	11.9
Pitressin	2 U	1	2	0.46	0.45	0.46	9.8
Pitressin	4 U	1	2	0.48	0.46	0.47	11.9
Pitocin	1 U	1	2	0.48	0.46	0.47	11.9
Pitocin	2 U	1	2	0.50	0.48	0.49	16.6
Pitocin	4 U	1	2	0.50	0.47	0.49	16.6

hormones stimulated creatine excretion. We are at a loss to explain why they should, on the one hand cause a quantitative transformation of creatinine into creatine, and on the other, cause a stimulation of creatine excretion.[1]

[1] Bloch and Schoenheimer (6) have shown that about 50% of creatinine containing N^{15} is excreted as such in the urine and that creatine isolated from the muscles did not contain any of the isotope. From these results they concluded that the biological transformation of creatine to creatinine was irreversible. Some of the reasons for the divergent results of the two laboratories are as follows.

Fifty per cent of the isotope in creatinine was not accounted for. This amount may have stimulated creatine excretion.

The increase in urinary creatinine was 75% of that fed showing a retention of 25% ,which may again have stimulated creatine excretion.

The extra amount of creatine, if any, formed in the muscles (irrespective of the fact that isotopic creatine was not isolated) was not determined colorimetrically. The isotope is only a "qualitative tag" so to speak, and for their conclusions to be valid it would be necessary to actually determine the total amount of creatine in both muscle and urine. This would then shift the emphasis from the isotope to creatine itself, since it is the physiological formation of the latter that is of most interest.

In our extensive studies with several hundreds of rats increases in muscle creatine last only 2 days after the precursor of creatine is injected. After this time the extra creatine is excreted as such into the urine. We believe that, in their study made at the end of 6 days after creatinine administration, if any isotopic creatine had been formed in the muscles up to 2 days it would have been excreted into the urine from 2 to 6 days. There would be no increase in muscle creatine during this time. Furthermore the New York investigators did not study urine creatine containing the isotope.

The dose of creatinine administered was not stated. We have observed (4) that it requires from 10 to 20 mg. of injected creatinine to cause an increase of 15 to 20% in muscle creatine accompanied by an increase of 48 mg. in extra creatine excretion. The 6-day experimental period is not, therefore, sufficient time to obtain the total amount of extra creatine excretion. We have found 2 to 3 weeks' time necessary for this purpose after the injection of a given substance such a creatinine or water.

The posterior pituitary hormones were not injected. The general metabolism of the body is constantly under the influence of all the hormones, including those of the posterior pituitary.

Their conclusions were drawn from results with only 3 animals. The New York workers do not seem to realize that large variations in creatine-creatinine metabolism may occur, and, while their positive results with the N isotope cannot be denied, nevertheless, their negative results are open to serious ques-

Thyroidectomy. Removal of the thyroid had no effect upon excretion of creatinine (fig. 6). The 20 mg. of extra creatine excretion was evidently due to the trauma and anesthesia of the operation during removal of the gland (8).

Desiccated thyroid. The injection of 0.1, 0.2, 0.3 and 0.4 mg. of this preparation dissolved in water caused increases in creatine excretion of 20, 21, 92 and 106 mg., respectively (fig. 6). The water content of the preparations injected could account for the first 2 values but not for the last 2. In these last 2 cases the excretion of extra

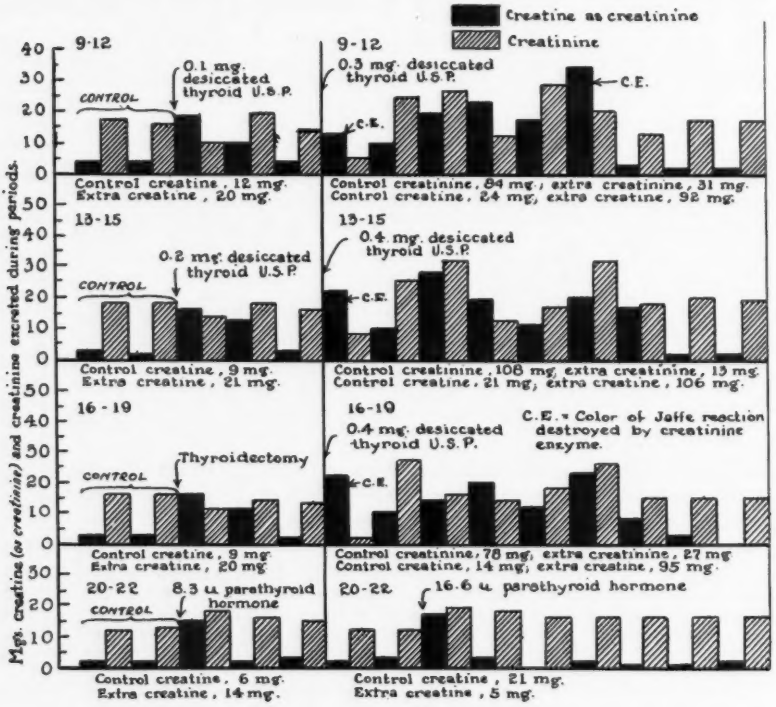

Fig. 6.

creatinine was 31 and 13 mg., respectively. These results confirm the well-known fact that, after thyroid stimulation, there is an increased metabolism in the body which results in an increased formation and excretion of both creatine and creatinine.

Thyroidectomy and desiccated thyroid. When 0.4 mg. of desiccated thyroid was injected into thyroidectomized animals the increased creatine excretion was 95 mg. and the increased creatinine excretion was 27 mg. (fig. 6). These values were too large to

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tion since we have shown with over 60 animals to date that the creatine→creatinine transformation does not take place in our experiments, while the creatinine→creatine transformation does occur.

The New York workers have shown that only a small amount of glycine containing N^{15} is utilized to detoxicate benzoic acid while most of the glycine that is used for this purpose comes from the body itself. It is, therefore, possible, as stated above, that creatine could also have been formed from body creatinine without respect to that containing N^{15}, since we have shown that creatinine stimulates creatine formation and excretion (4).

Hevesy (7), in discussing radioactive indicators, stated that, in the case of studies on radioactive phosphorus, the determination of both P^{32} and P^{31} content of the sample should be made.

be due to the trauma and anesthesia of the operation and it is, therefore, evident that the injection of this thyroid preparation caused an increased excretion of both creatine and creatinine in the normal and thyroidectomized animal.

Parathyroid extract. The creatinuria observed in this case was evidently due to the water content of the hormone injected (fig. 6). This hormone does not, therefore, influence creatine and creatinine excretion.

Zinc insulin and insulin. When 0.8, 1.6 and 3.4 U of Zn insulin (crystals) solution were injected the extra creatinine excretion was 127 and 75 mg. on the first 2 doses (fig. 7). The extra creatine excretion was 157, 110 and 48 mg., respectively. As the units of Zn insulin were increased the excretion of both creatine and creatinine progressively decreased, in fact on the 3.4 U dose 24 mg. of creatinine were retained.

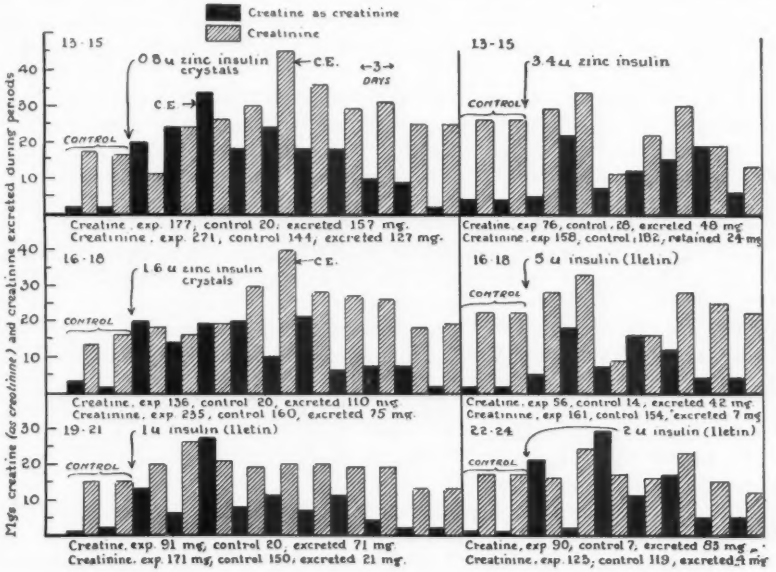
Creatine as creatinine
Creatinine
Mgs. creatine (as creatinine) and creatinine excreted during periods
13-15
0.8 u zinc insulin crystals
C.E.
←3→ DAYS
CONTROL
Creatine, exp. 177, control 20, excreted 157 mg.
Creatinine, exp. 271, control 144, excreted 127 mg.
13-15
3.4 u zinc insulin
CONTROL
Creatine, exp 76, control 28, excreted 48 mg.
Creatinine, exp 158, control 182, retained 24 mg.
16-18
1.6 u zinc insulin crystals
C.E.
CONTROL
Creatine, exp 136, control 20, excreted 110 mg.
Creatinine, exp. 235, control 160, excreted 75 mg.
16-18
5 u insulin (Iletin)
CONTROL
Creatine, exp 56, control 14, excreted 42 mg.
Creatinine, exp 161, control 154, excreted 7 mg.
19-21
1 u insulin (Iletin)
CONTROL
Creatine, exp. 91 mg, control 20, excreted 71 mg.
Creatinine, exp 171 mg, control 150, excreted 21 mg.
22-24
2 u insulin (Iletin)
CONTROL
Creatine, exp 90, control 7, excreted 83 mg.
Creatinine, exp. 125, control 119, excreted 4 mg.

Fig. 7.

When 1.2 or 5 U of insulin (iletin) were injected only small increases in creatinine excretion occurred (21, 4, 7 mg., respectively) while the extra creatine excretion was 71, 83 and 42 mg., respectively. The more pronounced action of the former preparation may have been due to its Zn content. It is possible that the action of these insulin preparations on creatine and creatinine excretion was due to their influence on sugar and protein metabolism, or both, since the creatinuria in these cases could not have been due to the breakdown of glycogen as postulated by Brentano (9).

SUMMARY

Parenteral injections of pitressin, with and without creatinine; pitocin; desiccated thyroid; parathyroid extract; insulin and zinc insulin, were made into normal rats, and, in the case of desiccated thyroid into thyroidectomized rats also, and the effect of this treatment upon creatine formation in the muscles and creatine-creatinine excretion in the urine was studied. In the case of the posterior pituitary hormones

particular attention was paid to their effect upon the creatinine→creatine transformation in the body. Results obtained were as follows.

Injection of creatinine stimulates the excretion of both creatine and creatinine in the urine.

Pitressin caused a stimulation of creatine excretion in some studies, and, after the injection of 4 U, a retention of creatinine was observed.

Injection of creatinine and pitressin together caused slightly more than one-third as much creatine excretion as was obtained when creatinine alone was injected. The creatinine retention after the injection of creatinine and pitressin was of about the same magnitude as the creatine excretion under these conditions.

After the injection of pitressin the body handles injected creatinine and that formed from protein metabolism in the body in a different manner. The former is largely excreted as such and stimulates the excretion of both creatine and creatinine in the urine while the latter is retained and is quantitatively transformed into creatine.

In 7 experiments the injection of pitressin in doses from 1 to 6 U caused a total retention of 324 mg. of body creatinine which was accompanied by an increased excretion of creatine, as creatinine, of 327 mg. In 4 experiments with 1 to 4 U of pitocin the creatinine retention was 223 mg. accompanied by an increased creatine excretion of 254 mg. From these results it is concluded that these hormones caused a quantitative transformation of body creatinine into creatine.

In 5 other experiments with these hormones little, if any, creatinine was retained. An increased creatine excretion of 22 to 150 mg. occurred. It is concluded that these hormones can also stimulate creatine excretion.

The injection of pitressin and pitocin caused increases from 10 to 17% in muscle creatine. Injections of the other hormones used in this study did not change the concentration of muscle creatine.

The creatinuria observed after thyroidectomy was probably due to the trauma and anesthesia of the operation.

Desiccated thyroid increases the excretion of both creatine and creatinine in the normal and thyroidectomized animal.

The creatinuria observed after injection of the parathyroid extract was probably due to the water content of the preparation.

Injections of zinc insulin caused a much larger excretion of creatine and creatinine than did similar doses of insulin, which may have been due to the zinc content of the former preparation. It is concluded that the effect of these preparations on creatine-creatinine metabolism was through their influence upon sugar and protein metabolism rather than to a breakdown of glycogen.

Reasons were given showing that the evidence offered by other investigators was insufficient for the conclusion that the creatine→creatinine transformation in the body is irreversible.

We wish to thank Doctor Oliver Kamm of Parke, Davis & Co. for the samples of pitressin and pitocin and Doctor J. M. Wilcox of E. R. Squibb & Sons for the parathyroid extract and zinc insulin solution.

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DIFFERENCE IN ACTION ON HEART BEAT COMPARED WITH THAT ON METABOLISM OF NORMAL AND TOXIC GOITER THYROIDS

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THYROIDECTOMY produces in man as in animals a decrease in metabolic rate that fairly uniformly amounts to about 30% of the original value. A concomitant reduction in heart rate is observed so universally that the conclusion seems to be justified that both symptoms are manifestations of one and the same deficiency of the thyroid hormone. The observation that thyroid medication stimulates metabolic rate and, in a similar way, heart rate, supports the assumption of the identical etiology.

Parallelism has been claimed to such an extent that some authors recommended the counting of the pulse rate as a substitute for determination of basal metabolism. The observation, however, of hypothyroid as well as of hyperthyroid cases with normal or even paradoxical heart rates and the fact that some hypothyroid patients show 'low tolerance' to thyroid, developing tachycardia and nervous symptoms on medication with thyroid substance before the metabolism reaches normal levels, are not in accord with that theory.

To account for such exceptions two explanations have been offered; the supposition of a duplicity of thyroid hormones and that of an occasional formation of a hormone of abnormal composition with increased toxicity. Such hypotheses, derived from observations on patients, have remained controversial, and experimental evidence in support of either viewpoint has not been forthcoming.

The complete absence of any functioning thyroid tissue in the organism to be used for such investigation appears an absolute necessity and the difficulty of finding suitable human material for that purpose is quite obvious.

Of thyroidectomized animals it was found that the male adult rat is an appropriate testing object for both metabolic rate and heart function (1–2). We were able to demonstrate a relative independence of both effects and a variability of response which depended on the type of thyroid product administered. The metabolic rate of small animals is more accurately expressed in relation to body weight than to surface. For that reason the metabolism as determined in our previous work, has been measured by mg. oxygen consumed per hour calculated on kg. body weight. Measured by that method, the normal albino rat gives figures from 1400 to 1600. The normal heart rate is 300 per minute or slightly above.

After thyroidectomy, 6 to 8 weeks are required until the animal is adjusted to the athyroid level which corresponds to metabolic values from 900 to 1200, usually below 1150, and heart rates of 190 to 200.

Restoration of the thyroidectomized rat to normal condition would mean an increase of metabolism by 45% to 50% and corresponding augmentation of the heart beat by 50%, or 100 beats per minute. If the thyroid action were dependent on one

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and the same substance, then parallel effects should be obtained in every case provided adequate dosage was given.

In accordance with the technic used in the tests described in detail elsewhere (1), the animal was thoroughly standardized and the medication calculated per 10 gm. of body weight was administered by stomach tube on 3 consecutive days. The metabolic and cardiac responses were determined on the fifth day.

In testing various thyroid preparations it was found that effect on heart rate and metabolism as a rule do not run parallel. If crystallized commercial thyroxin was used a 50% increase in metabolic rate corresponded to a heart rate stimulation of about

TABLE 1. PIG THYROIDS

(Iodine percentages are determined in the fresh gland)

Material	Iodine, γ in dose per 10 gm. weight	O_2 Consumption mg./hour/kg.			Heart rate	
		Before	After	% increase	Before	After
I. Pulp emulsion 0.08% iodine	0.38	935	1105	18	195	205
	0.38	935	1205	29	194	200
	0.38	910	1250	37	190	200
	0.62	930	1295	37	189	198
	0.62	950	1325	40	195	200
	0.62	950	1305	38	195	205
	1.32	940	1440	53	195	212
	1.32	880	1250	42	185	205
	1.32	920	1260	37	193	205
Pieces of same thyroid	2.70	1000	1615	62	192	225
II. Thyroid 0.087% Iodine	1.70	1000	1625	62	196	225
	1.37	1110	1475	33	197	240
III. Thyroid 0.122% iodine	4.00	950	1495	56	196	260
	2.93	905	1415	55	196	289
	2.60	950	1500	56	197	295
IV. Thyroid 0.15% iodine	3.30	950	1580	66	195	212
	7.50	1025	1545	51	194	198
	5.80	1030	1695	65	190	225
	4.00	1030	1290	25	194	200
Same thyroid dried	2.00	965	1520	56	196	205
	1.70	900	1245	38	198	212
	2.45	1020	1560	54	197	208
	2.24	925	1415	53	194	215

50 beats instead of 100. By adjusting the dosage to effect a metabolic increment of 30%, which lends itself better to comparative studies, the acceleration of the heart was reduced to 30 beats per minute. Thyroxin purified in our own laboratory seemed to be almost free from any effect on cardiac frequency (3). A similar weak action on the heart rate was observed with thyroid globulin, while some commercial U.S.P. thyroid preparations produced a tachycardia up to 400 beats per minute with a 30% metabolic stimulation. This characteristic of U.S.P. thyroid was observed fairly generally, but the degree varied widely, some brands showing the effect on the heart almost in proportion with the calorigenic effect, or at least only slightly exceeding it (4). Hydrolysis of thyroid globulin, of low cardiac effect, produced split products having a striking action on the heart rate. This suggested the probability that the marked effect on the heart shown by dried thyroid powder might be due to autolytic cleavage occurring in the glandular material before the drying process was sufficiently advanced.

To elucidate this question, the effect of feeding fresh pig's glands on thyroidectomized rats was studied. The maintenance of an accurate dosage of fresh glandular material is difficult in view of the small quantities which are given. The structural fibrous elements cannot be ground to sufficient fineness to permit a uniform suspension. An emulsion of the gland in tragacanth gum solution was first tried, for which purpose the tissue was scraped with a knife and passed through a fine sieve. Definite quantities of the suspended material were given, as usually done before, on 3 days by stomach tube. Later on, simply small pieces of the gland were fed. The latter method had the advantage that the animals ate the material willingly.

TABLE 2. NORMAL HUMAN THYROIDS

Material	Iodine, γ in dose per 10 gm. weight	O_2 Consumption mg./hour/kg.			Heart rate	
		Before	After	% increase	Before	After
I. Thyroid fresh	1.92	965	1400	45	192	212
	3.02	980	1470	49	194	200
	0.60	900	1170	30	191	200
	0.86	1000	1410	41	194	232
Same thyroid dried	5.70	1040	1585	52	194	216
	2.60	1020	1490	46	194	240
	1.50	1080	1280	19	199	205
	4.20	1065	1525	43	193	215
	0.94	1000	1360	36	198	225
II. Thyroid fresh	2.24	900	1345	50	195	225
	0.95	875	1190	36	194	210
	1.98	950	1450	52	192	208
	0.86	995	1205	26	195	214
Same thyroid dried	4.00	910	1370	50	193	225
	3.00	1000	1530	53	192	225
	6.00	1100	1750	59	193	222
	1.60	1065	1390	30	199	224

I. Supposedly normal thyroid from autopsy of man about 50 years old, killed by subway train by accident or suicide. Iodine content 0.064% in fresh gland.

Dr. Marine's findings: Gland shows in some parts signs of beginning autolysis, evidenced by desquamated epithelium. Follicles are uniform in size. Generally normal thyroid.

II. Supposedly normal thyroid from 12-year-old boy, killed in accident. Iodine content 0.086% in fresh gland.

Dr. Marine's finding: Slight signs of beginning autolysis. Otherwise normal gland.

In view of the difficulties of dosage the quantities of iodine in the daily medication, as given in the tables, must be considered as rough approximations. Thyroid, and especially the diseased gland, is hardly so uniform in structure that one should expect a small particle of the gland to always contain the same percentage of iodine as that found by analysis of a larger piece. Therefore, it is not surprising that low dosage occasionally produces strong calorigenic effects and vice versa. What is of interest is essentially the proportion between metabolic increment and the effect on heart rate.

Table 1 represents the results obtained with fresh pig's glands. The first gland showed consistently an almost complete absence of any action on the heart rate. Gland II seemed to have some effect but the number of tests is insufficient. In Gland III the action on the heart was noticeable. Unfortunately, no sections had been prepared from that gland to ascertain whether or not any pathologic conditions might be present. Gland IV again was devoid of cardiac stimulation.

The difference between the Glands I and IV on one side and III on the other is sufficiently marked to raise the question as to the underlying cause. This led to the extension of the study on human glands.

TABLE 3. PATHOLOGIC HUMAN THYROIDS

Material	Iodine, γ in dose per 10 gm. weight	O_2 Consumption mg./hour/kg.			Heart rate	
		Before	After	% increase	Before	After
Thyroid III	1.22	1000	1450	45	190	245
	1.20	1015	1275	27	190	275
	1.48	850	1130	33	195	265
	0.74	1070	1160	8	190	245
	1.36	1100	1460	33	194	270
	1.22	1070	1380	29	195	257
	1.01	860	1080	25	190	280
	1.85	1000	1270	27	193	258
Thyroid IV	0.74	990	1070	8	194	305
	0.67	1100	1535	40	194	277
	1.26	1060	1060	0	197	300
	0.58	900	1145	27	195	281
	0.32	1100	1315	20	196	280
	0.62	960	1280	34	196	300
Thyroid V	2.00	950	1450	53	200	300
	1.19	985	1375	40	198	300
	1.11	950	1150	21	197	315
	3.20	1030	1570	52	196	325
	4.10	940	1535	63	194	340
	0.69	860	1050	22	220[1]	315
	0.38	1120	1230	10	195	245
	0.59	970	1275	31	195	277
Thyroid VI	1.59	890	1215	36	192	300
	0.90	905	1255	39	196	330
	3.80	1000	1460	46	194	360
	0.33	1170	1170	0	196	310
	0.75	900	1120	25	198	295
	0.60	840	1135	35	195	315
	2.70	1000	1170	17	205	385
Thyroid VIIa	2.80	1020	1420	40	196	305
	3.84	1000	1650	65	197	310
	1.32	940	1290	37	200	330
	0.80	1040	1180	13	198	300
	0.76	900	1185	31	196	295
	1.48	1100	1270	16	197	325
	0.93	1100	1130	3	195	288
Thyroid VIIb (2nd lobe)	0.48	1030	1300	26	192	260
	0.28	1100	1275	16	200	300
	0.54	990	1215	23	193	265
	1.92	980	1460	49	195	300
	1.15	1065	1425	34	195	277
Thyroid VIII	0.42	950	1390	46	195	250
	0.42	1050	1320	26	193	300
	0.99	1055	1255	19	199	290
	0.78	1105	1440	30	193	295
Thyroid IX	0.55	1160	1535	32	195	240
	0.95	880	1120	27	195	205
	0.38	955	1095	15	196	222
	0.33	930	1085	17	198	212
	1.10	1000	1480	48	194	230
	0.40	990	1210	22	197	218
	2.15	900	1360	50	190	240
	1.40	920	1330	44	198	225

[1] The initial heart rate in this rat was the highest we have encountered in a control animal, but the low metabolism speaks against incomplete thyroidectomy.

Pathologic thyroids were obtained from the College of Physicians and Surgeons, New York Hospital, and New York Post-Graduate Hospital.[1] For comparison, 2 supposedly normal glands from accident cases were obtained from the Medical Examiner's Office.

All glands, as soon as possible after removal, were placed in the freezing unit of the refrigerator and kept frozen until used. The pathologic glands were accompanied by the pathologist's report, and, from the patient's history, were known to originate from cases of clinical hyperthyroidism. Additional sections were prepared from the glands after arrival in our laboratory.

We are indebted to Dr. David Marine of Montefiore Hospital, The Bronx, for his courtesy in giving us his evaluation of the slides submitted to him. Dr. Marine's report is included in each case.

With the view of rendering the tests more convincing, the dosage has been varied widely. The object was to show either presence of heart stimulation at low metabolic response or its absence at high metabolic increase.

Description of Thyroids Reported in Table 3

III. Diagnosis was that of toxic diffuse goiter; iodine content in fresh gland was 0.037%. *Pathologic report.* Gross. The specimen consists of 3 small fragments of thyroid tissue, the largest measuring 1.5×0.6×0.5 cm. These are reddish-yellow, homogeneous, translucent, and a very small amount of colloid is on the surface. Microscopic: The follicles of the thyroid vary only slightly in size and shape, the majority are small, regular, and well filled with colloid which shows pronounced marginal vacuolization. The lining cells are low columnar to cuboidal, and papillary projections into the lumen are present. The stroma is scanty. Scattered collections of lymphocytes and occasional lymph follicles are present. Histologically this is a stage of late involution. *Dr. Marine's report.* Slight lymphoid infiltration; colloid slightly reduced but present in all follicles, the latter fairly uniform in size. No fibrosis; no infoldings. Epithelium high cuboidal. Shows effect of iodine medication. Résumé: colloid—early moderate hypoplasia (involuting).

IV. Diagnosis was that of toxic diffuse goiter; Iodine content in fresh gland was 0.023%. *Pathologic report.* Follicles lined with high columnar cells, numerous papillary infoldings, marginal vacuolization of colloid material and occasional lymphocytic infiltration. *Dr. Marine's report.* Interlobular stroma slightly increased. Few small lymphocytes. All follicles lined with columnar epithelium with marked infoldings; colloid greatly reduced; larger follicles containing thin vacuolated colloid. Moderate marked hypoplasia.

V. Diagnosis was that of toxic diffuse goiter; Iodine content in fresh gland was 0.054%. *Pathologic report.* Gross: The specimen consists of a portion of thyroid gland weighing 2 gm. and measuring 2.5×2×0.6 cm. One surface which is covered by a thin capsule is reddish-pink. The other surfaces are pink-gray and finely lobulated and meaty. Microscopic: Sections through the thyroid gland reveal acini which vary in size from the very small to the medium sized. They are uniformly lined by cuboidal to flat cells. The acini are filled with colloid and there is a moderate amount of marginal vacuolization. There are scattered intra-acinar epithelial proliferations. There are scattered lymph follicles. There is some increase in the interlobular connective tissue. Sections show a marked degree of involution. *Dr. Marine's report.* Beginning adenoma formation, increase of stroma, slight increase in lymphoid cells; follicles varied in size; large follicles with flat cuboidal epithelium, significant for recent iodinization. The small follicles still with high cuboidal and low columnar epithelium. Colloid—early hypertrophy.

VI. Diagnosis was exhaustion atrophy of the thyroid in hyperplastic goiter. Iodine content in fresh gland was 0.024%. *Pathologic report.* Gross: This is a lobe of thyroid measuring 5×4×1.5 cm. in size. It weighs 16 gm. The parenchyma is unusually firm and white and there seems to be no doubt that it is hyperplastic. Microscopic: This bit of thyroid is very interesting in that the epithelial cells show a marked metaplasia which is almost precancerous. These cells are gathered together in small nodules where an occasional lumen may be seen. They are cuboidal in nature and appear to be proliferating wildly. Occasional mitotic figures are noted. Throughout the whole section there are many well localized areas of lymphocytes. There are some large acinar formations lined by cuboidal epithelium and filled with immature colloid. *Dr. Marine's report.* Fibrosis; numerous lymphocytes; exhaustion atrophy.

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[1] We express our thanks to Dr. W. W. Palmer for securing for us Thyroids III, IV, and V to Dr. Ephraim Shorr for Thyroids VI, VII and VIII and to Dr. J. William Hinton for Thyroid IX.

VIIa. Diagnosis was hyperplastic goiter. Iodine content was 0.04% in fresh gland; H_2O, 80.5%; thyroxin, 0.0052% which equals 0.0034% thyroxin iodine. (The thyroxin iodine represents only 8.5% of the total iodine, as compared with about 25% in normal glands. This gland was the only one of a size sufficient to permit the thyroxin determination.) *Pathologic report.* Gross: The specimen is a large thyroid lobe 9×5×3 cm. in size and weighing 68 gm. The section surface is coarsely nodular, light-brown with a tinge of yellow. Microscopic: This thyroid contains a few diffuse areas where the epithelial cells show evidence of marked proliferation. Here they are somewhat columnar in nature and definite spur-formations are observed. On the whole the gland is well involuted, but the colloid is immature, with scalloped edges. Throughout the section several collections of lymphocytes may be found. *Dr. Marine's report.* Fibrotic gland; intralobular sclerosis; colloid greatly reduced; follicles small.

VIIb. Diagnosis was adenomatous hyperplasia of the thyroid. This gland is the second lobe of Gland VIIa and was removed two weeks later. During this time the iodine content increased to 0.061%. *Pathological report.* Gross: This is a lobe of thyroid which is said to be the right. It measures 4×7.5×4.5 cm. and weighs 45 gm. On section it is found to be lobular and rich in colloid as though the involution had been good. It contains an occasional adenomatoid mass. Microscopic: This section contains cysts lined by flat epithelium and containing immature colloid. In several areas these hyperplastic epithelial cells are arranged in small groups with miniature lumina. In one or two areas the epithelial lining is arranged in spurs and tends to be columnar in nature. There is only a slight increase in the fibrous tissue. *Dr. Marine's report.* Interlobular stroma is increased and contains a few small foci of lymphoid tissue. The follicles in general are small, fairly uniform in size and colloid containing throughout. The epithelium is cuboidal in all follicles, indicating moderate activity. Diagnosis: Early-moderate hyperplasia, involuting from a more advanced hyperplasia.

VIII. Diagnosis was that of degenerative adenomatious goiter. Iodine content n fresh gland was 0.051%. *Pathologic report.* Gross: The specimen is a large flabby mass said to be from the thyroid accompanied by a small punctured cyst. The large mass measures 8 cm. in length and 4.5 cm. in diameter. From one pole water blood exudes. The specimen weighs 85 gm. On section the mass is found to be composed of interlacing cords of fibrous tissue, numerous cysts and broken down adenomatous material. The cyst wall is apparently nothing but fibrous sheets 3.5×3 cm. in area and about 2 mm. in thickness. It is noted that it is filled with glistening particles of cholesterol. Microscopic: This section shows many miniature acini lined by cuboidal epithelium cells and filled with immature colloid. Scalloping around the edges may be seen and several large cysts are also present. Groups of these acini are separated by strands of fibrous tissue. Many areas of degeneration are scattered about and the picture is typical of a fetal adenoma. *Dr. Marine's report.* The interlobular stroma is only slightly increased. There is a marked increase in the number and size of the lymphoid foci throughout. The follicles are moderately irregular in size and all show infoldings of the lining epithelium. All follicles contain pale, uniform colloid. The epithelium in general is high cuboidal and evidently involuting from a more advanced degree of hyperplasia. Diagnosis: Moderate hyperplasia, involuting.

IX. Diagnosis was that of nodular goiter with hyperthyroidism (recurrent). Iodine content in fresh gland was 0.05%. *Summarized history.* A 44-year-old white female was admitted with a complaint of a lump in the neck which developed about 6 months ago. Thyroidectomy for toxic goiter had been performed 7 years before. The present symptoms included all manifestations of hyperthyroid disease. B.M.R.+34. The patient was treated with iodine for three months but did not receive further iodine medication during the months preceding the second thyroidectomy. *Dr. Marine's report.* Histologically the changes are fairly complex so that it is not possible to classify it within the simple cycle. In general there is a diffuse adenomatosis, the adenomata being small and ill defined, and some are composed of large colloid containing follicles, other adenomata are papillomatous, still others are composed of oxyphilic (Hurthle) cells, and one adenoma suggests the possibility of a premalignant change. There are numerous coarse stroma bands containing large numbers of lymph foci. In general the colloid is reduced and the epithelium is somewhat active. In our classification I would group it as a diffusely adenomatous goiter in the stage of early hypertrophy, but of course one cannot say whether it has regressed from a more marked hyperplasia or is progressing from a colloid or resting stage. The chances are that during the life history of the goiter there have been several periods of involution and hypertrophy.

DISCUSSION

Comparison of the various tables shows strikingly that the normal glands had only a slight action on the heart rate, whereas the III to VIII hyperthyroid glands uniformly produced a conspicuous stimulation. The logical conclusion would be that the hyperthyroid gland contains a substance that specifically stimulates the heart rate. We were about to draw that conclusion, when Thyroid IX became available. The patients of Cases III to VIII had received the usual iodine treatment preceding the operation. In Case IX the iodine had been omitted during the last months.

The difference in the action on heart rate of this latter thyroid is striking; the cardiac effect, although not as weak as in the normal glands, was considerably below that observed with the 6 other glands. One must be chary in forming theories based on observations of a single case and if we try to present possible explanations we do so with the necessary reserve. It could be pure coincidence that the non-iodized thyroid showed a reduced action on the heart rate. The thyroids in hyperthyroidism are not always identical in their pathology and might also be different in physiologic effect. This would mean that the iodine treatment had nothing to do with the difference in effect on the heart rate.

The proof or refutation of this assumption can be brought out only by future research which will take time in view of the infrequent opportunity of obtaining such non-treated thyroids. Another explanation is based on the theory that "in toxic goiter the primary fault is a too easy escape of hormone from the gland" (5), and that such leakage is stopped by iodine treatment. Assuming the production of a substance in the thyroid responsible for specific action on the heart rate, it can be visualized that the iodine treatment leads to an increased storage of that substance within the gland. To what extent such substance is responsible for other by-effects, especially for nervous symptoms, remains to be investigated.

It is of interest to note that in Case VII the iodine content between first and second stages of operation increased markedly. It would be of value to know whether newly deposited iodine possesses full calorigenic activity. In commercial desiccated thyroid an average of 0.62 γ iodine per 10 gm. given for 3 days produces a 30% metabolic increase. Lower and higher responses are not suitable for comparison. Medication with the same quantity of iodine in the form of the glandular substance might have given some information on this subject. This, however, was not feasible. Besides the possible inaccuracies in dosage inherent in the use of fresh glands, there seems to be a considerable degree of variability in the composition of various parts of a gland as evidenced by the fact that in some instances a fairly high dosage calculated on iodine content showed little metabolic response in contrast to parallel tests with material from the same gland.

The question may be raised as to how an increase in metabolism can be reconciled with a sustained bradycardia. The answer is that apparently the cardiac output was augmented. Although this cannot be demonstrated objectively with figures, it was noted that the heart beat was stronger and more distinct in every instance in which there was an increase in metabolism. Such a change was not observed when the heart rate was increased unless it was accompanied by a substantial increase in metabolism.

A high grade of similarity between the histologic picture of the normal glands and that of VIIb was observed. Since a difference in physiologic function of both glands is evident from the tables submitted, it shows that the histologic picture does not reflect every pathologic process in the gland.

In our previous publications we expressed the opinion that the strong cardiotropic effect of the commercial dried thyroid substance might be due to postmortem changes, for example by autolysis. We were led to this assumption by the fact that thyroid globulin, which has only a slight effect on cardiac rate, forms by hydrolysis split products of enormously increased effect on the heart. We tried to verify that surmise.

Parts of pig's Gland IV and parts of the two normal human glands were kept at room temperature for 5 hours and then slowly dried at 37° C. In the case of human Gland I some water was added again to prolong the time of autolysis and bacterial growth. The dried material was retested. The results are included in the tables of the 3 glands. There was no difference in action as compared with the fresh gland. Autoly-

sis does not seem to affect the thyroid hormone in such a way as to change its physiologic action.

It is presumed, therefore, that the difference in action of the commercial thyroid must be found in the original glandular material. Possibly a relatively large percentage of pathologic glands is processed in the manufacture of dry thyroid, or the feeding of iodine to the animals, which are the source of the glands, as practiced in large parts of the country and which may often be excessive, may have some influence. We found iodine values up to 0.3 % in fresh pig's gland which corresponds to about 1.2% after drying; that is 6 times the U.S.P. XI strength.

SUMMARY

Fresh human and pig thyroids were fed to thyroidectomized rats and the effect on heart rate and metabolism compared. Pig's gland showed either a slight or moderate stimulating effect on the heart rate as compared with the simultaneous effect on the rate of metabolism. Normal human glands had a slight action on the heart, whereas thyroids removed surgically from patients with toxic goiter, which had been treated with iodine, stimulated the heart rate to a variable but always quite marked degree, which in some instances was far greater than would be expected from the concomitant effect on metabolism. A single thyroid from a patient with toxic goiter, where iodine was not given preceding operation, had a weaker action on the heart rate. Subjecting the glands of weak cardiac action to autolysis did not alter the quality of physiologic activity.

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FURTHER STUDIES ON THE OVARIAN-THYROID RELATIONSHIP[1]

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MANY METHODS have been used to study the relation of the ovaries to the thyroid glands. Van Horn (1) reported that three rat units of theelin were necessary to produce estrus in experimental hyperthyroid rats. Gessler (2) reported a decrease in basal metabolism in guinea pigs with the use of folliculin. Collett, Smith and Wertenberger (3) found that theelin, theelol or amniotin increased the basal metabolism of ovariectomized women. Sherwood et al. (4) showed that theelin and amniotin lowered the basal metabolism of ovariectomized rats. The author also showed in the above investigation that amniotin lowered the basal metabolic level in experimental hyperthyroid rats. Grumbrecht and Loeser (5) investigated the relation of ovarian to thyroid function through a study of structural changes in the thyroid gland. They suggested that ovariectomy caused a flattening of the epithelial cells and allowed a storage of colloid. It is suggested that the thyrotropic hormone was increased as a mechanism of defense against the future decreased activity of the thyroid. Large doses of estradiol monobenzoate were used in the above investigation to show a decrease in oxygen consumption.

In view of the above findings, it seemed desirable to determine the effect of amniotin and estradiol upon the basal metabolism of intact and operated rabbits before proceeding with further studies.

EXPERIMENTAL

The animals were maintained at a temperature of 26°C. during the entire procedure. It was observed that the animals were less active and gave more consistent results at the above mentioned temperature. The diet which consisted of Purina Chow was fed throughout the experiment. All animals were kept in the experimental room one month prior to the period of investigation.

A modification of the Haldane apparatus (6) was used for the determination of basal metabolism. Individual tests were run for a period of 1 hour following a 22-hour fasting period. The basal metabolism was determined during the hours 10 A.M. and 4 P.M. since the animals were less active during those hours.

Ten of the 22 animals were thyroidectomized and basal metabolism determinations made until a definite hypothyroid level was obtained. A consistent hypothyroid level was not observed in less than 28 days following thyroidectomy. At the end of this period 4 of the animals were given 100 mg. of desiccated thyroid per kg. The thyroid substance was given daily for 3 days. The maximum basal metabolism was found and the experiments continued until the hypothyroid level had again been established. This group was used as a control for the potency of the desiccated thyroid.

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Six rabbits were used for the study of amniotin and estradiol. Each animal of this group received 100 mg. of desiccated thyroid per kg. per day for a period of 3 days. Upon the cessation of thyroid feeding oil solutions of estradiol and amniotin were injected subcutaneously. Three animals received 2000 I.U. of amniotin daily for a period of 4 days. The remaining 3 animals received 5 γ of estradiol daily for a period of 4 days.

RESULTS

Table 1 presents the data which are typical of the findings in the present investigation. The weight, percentage decrease in metabolism due to thyroidectomy, percentage increase in basal metabolism due to thyroid therapy and the number of days necessary for the animals to return to the hypothyroid level are given.

TABLE 1. EFFECT OF ESTROGENIC HORMONES UPON HYPERTHYROID RABBITS

Animal	Weight	Decrease in B.M.R. due to thyroidectomy	Increase in B.M.R. due to thyroid therapy	Return to hypothyroid level
	gm.	%	%	days
		Thyroid Controls		
1	3480	35.5	80.9	36
2	3700	39.5	71.7	35
3	3670	38.0	75.6	30
4	2750	48.0	84.2	36
Average	3400	40.2	78.1	34
		Experimentals		
5	3210	39.1	64.1	17
6	3450	44.7	66.2	16
7	3640	43.4	80.0	19
8	3310	40.0	60.0	17
9	3710	41.2	72.0	16
10	3000	43.1	71.6	13
Average	3386	41.9	68.9	16

The thyroid controls show a decrease of 40.2% following thyroidectomy and an increase of 78.1% above the hypothyroid level following thyroid feeding. These animals returned to the hypothyroid level in 34 days.

The experimental animals show a decrease of 41.9% following thyroidectomy and an increase of 68.9% above the hypothyroid level following thyroid feeding. These animals returned to the hypothyroid level in 16 days. Animals 5, 6 and 7 received 5 γ of estradiol daily. Animals 8, 9 and 10 received 2000 I.U. of amniotin daily.

Protocol 1 shows a maximum caloric output 48 hours following the cessation of thyroid feeding and a return to the hypothyroid level in 36 days. Protocol 2 shows a maximum heat production 48 hours after thyroid feeding and a return to the hypothyroid level in 17 days.

DISCUSSION

The author, in a previous publication (4), stated that estrogenic substances might inhibit thyroid function by way of the hypophysis. Later Grumbrecht and Loeser (5) suggested that the thyrotropic principle of the hypophysis was inhibited when estrogens were administered. Their conclusions were based upon a correlation between structural changes of the thyroid and a decrease in basal metabolism. Contrary to the above findings, Sherwood et al. (7) found a marked structural change in the

PROTOCOL 1
Rabbit 4. Basal metabolism

Date	Weight	Oxygen	R.Q.	Calories per kg. per hour	Remarks
	gm.	gm.			
8/16/39	2141	1.76	0.86	2.81	Normal
8/29/39					Ovariectomy
9/11/39					Thyroidectomy
9/16/39	2321	1.81	0.80	2.61	
10/11/39	2879	1.20	0.97	1.46	
10/24/39					Desiccated thyroid 100/mg./kg.
10/25/39					Desiccated thyroid 100/mg./kg.
10/26/39	2772	1.85	0.85	2.26	Desiccated thyroid 100/mg./kg.
10/27/39	2617	1.65	0.81	2.12	
10/29/39	2551	2.00	0.89	2.69	
10/31/39	2566	1.85	0.88	2.46	
11/ 3/39	2679	1.80	0.90	2.31	
11/ 8/39	2815	1.70	0.92	2.08	
11/13/39	3039	1.75	0.91	1.98	
11/29/39	3143	1.80	0.83	1.93	
12/ 1/39	3152	1.40	0.96	1.54	Hypothyroid level 36 days
12/ 7/39	3269	1.45	0.98	1.55	

PROTOCOL 2
Rabbit 9. Basal metabolism

Date	Weight	Oxygen	R.Q.	Calories per kg. per hour	Remarks
	gm.	gm.			
8/25/39	2055	1.57	0.89	2.69	Normal
8/28/39	2063	1.63	0.76	2.68	Normal
8/29/39					Ovariectomy
9/23/39	2384	2.05	0.76	2.85	
10/29/39	2671	2.10	0.83	2.65	
11/16/39	2665	2.18	0.88	2.79	
12/ 4/39	2644	1.95	0.97	2.58	
1/20/40	2675	1.85	1.00	2.43	
2/22/40	2733	2.00	0.84	2.47	
2/24/40					Thyroidectomy
3/20/40	3707	1.40	0.94	1.31	
3/22/40	3718	1.80	0.75	1.60	
3/25/40	3718	1.70	0.86	1.55	Desiccated thyroid 100/mg./kg.
3/26/40					Desiccated thyroid 100/mg./kg.
3/27/40					Desiccated thyroid 100/mg./kg.
3/28/40	3530	2.15	0.83	2.05	Estradiol 5γ
3/29/40	3431	2.55	0.76	2.46	Estradiol 5 γ
3/30/40	3346	2.00	0.78	1.99	Estradiol 5 γ
4/ 1/40					Estradiol 5 γ
4/ 6/40	3023	1.80	0.86	2.02	
4/ 8/40	3083	1.78	0.77	1.92	
4/10/40	3151	1.78	0.83	1.90	
4/12/40	3282	1.86	0.88	1.93	
4/13/40	3295	1.36	1.00	1.45	Hypothyroid level 17 days
4/19/40	3361	1.42	0.95	1.47	
4/24/40	3403	1.33	0.90	1.34	

thyroid glands but no measurable change in the basal metabolic rate. These results have been confirmed by many previous investigators.

The author believes that the existing data is insufficient for the clarification of the relationship that exists between the thyroid and ovaries. Grumbrecht and Loeser

(5) believe that the secretion of thyrotropic hormone is limited due to the ability of sex hormones, administered parenterally, to inhibit this function of the hypophysis. The author suggests that a metabolic principle might be secreted by the hypophysis and that this may be affected by the estrogenic substances. The thyrotropic principle of the hypophysis could not have been of concern in the present investigation since the thyroid glands had been removed. Adequate data have not been presented to show that an antihormone reaction might have been responsible for the observed results.

Similar results have been obtained when theelin, amniotin, stilbestrol and estradiol were used in the control of basal metabolism. These hormones were effective in the reduction of the basal metabolic rate of rabbits and rats. An increase of approximately 75% in basal metabolism was obtained with the administration of 100 mg. of desiccated thyroid per kg. Ten times that amount was necessary to produce the same effect in rats.

Since no definite metabolic principle has as yet been reported for the hypophysis and the principal glands related to metabolism control were removed, it is considered possible that a direct tissue effect was obtained in the present investigation. Many investigators have been able to change the rate of oxidation of tissues in vivo as well as in vitro. One might therefore expect a similar effect in the present investigation without pituitary intervention. It is therefore suggested that a chemical study of the tissues and their waste products be made as a final solution to the relationship existing between the thyroid and the ovaries.

SUMMARY

The thyroid-ovarian relationship of the rabbit is comparable to that of the rat when measured by means of basal metabolic changes. Estrogenic substances lower the basal metabolism of rabbits.

The author wishes to express thanks to Dr. E. L. Sevringhaus for his generous suggestions and criticism during the period of investigation, and to Dr. J. A. Morrell of E. R. Squibb & Sons for the generous supply of amniotin.

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THE EFFECTS OF FRACTIONATED CHORIONIC GONADOTROPIC EXTRACT ON SPONTANEOUS ACTIVITY AND WEIGHT OF ELDERLY MALE RATS

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As MENTIONED in previous publications (1, 2), several estrogens have been found to increase the levels of spontaneous activity in elderly rats. The augmented activity has commonly been found to be associated with considerable losses of weight and the question was raised whether the disturbed nutrition rather than the induced hormonal imbalance was the cause of the increase. In short, did it represent merely something akin to the restlessness of an ailing child?

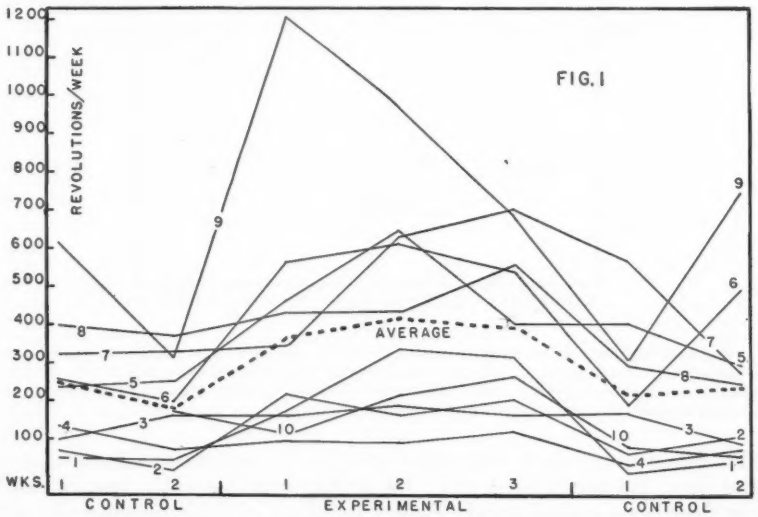

Fig. 1. EFFECTS ON ACTIVITY LEVELS OF ESTROGENIC FRACTION OF EMMENIN ADMINISTERED TO ELDERLY MALE RATS. The extract was given for 3 weeks preceded and followed by control periods of 2 weeks each.

One of the estrogens that has been utilized in our investigations is chorionic gonadotropin marketed under the trade name, emmenin. Through the kindness of the manufacturers of the product[1] we have recently had opportunity to test two steroid-containing derivatives, one of which had been found to be estrogenic and the other not. Tests of these materials threw some light on the relationships of estro-

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[1] Our thanks are due to Ayerst, McKenna & Harrison for supplies of fractionated emmenin.

genicity, on the one hand, and nutrition-disturbance, on the other, to the activity levels.

As in the preceding studies, the rats were kept in self-recording activity cages and given the emmenin fractions in a paste made with cracker-meal. The dosage, after a few exploratory tests, was arbitrarily set at a relatively low level of 1 cc., three times per week. This sufficed to bring out the characteristic weight losses and, in the active fraction, to influence significantly the activity levels. Ten experiments were made with the estrogenic fraction and 13 with the non-estrogenic.

The essential features of the experiment are apparent from the accompanying graphs. In figure 1 it is seen that the estrogenic fraction, with one dubious exception,

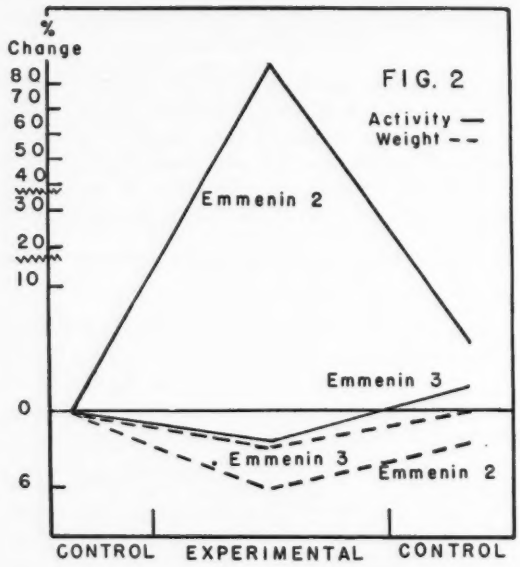

Fig. 2. Effects of 2 fractions of emmenin on activity and on weight. Emmenin 2 was estrogenic and emmenin 3 non-estrogenic. Changes expressed as percentile variations from initial levels. "Control" values are means of averages for 2 weeks: "Experimental" values are means of averages for 3 weeks. Note difference in scale values for top and bottom portions of graph.

led to consistent increase in the levels of activity. The non-estrogenic fraction, on the other hand, had no consistent effect, the activity levels varying in random fashion from those of the control periods. As seen in figure 2, the average activity level for the estrogenic fraction increased 89% over the pre-treatment level, whereas that for the non-estrogenic fraction was essentially the same in both periods. With the dosages that were used, the weight changes were not great but for the estrogenic fraction there was an average decrease of 6% from the initial control weight. The weight loss from the non-estrogenic preparation was half as great. It is evident therefore that while both fractions led to weight loss, only the one which had estrogenic properties caused augmentation of activity. The data thus add support to the hypothesis that the increase of spontaneous activity that results from the administration of estrogens is due to a hormonal influence rather than to non-specific interference with nutrition.

SUMMARY

In extension of previous studies two fractions of chorionic gonadotropic extract were administered to elderly male rats kept in self-recording activity cages.

An estrogenic fraction caused increase of activity as well as weight loss.

A non-estrogenic fraction caused loss of weight but the activity levels showed only random variation.

The activity-increasing effect of estrogenic hormones in elderly males seems therefore to be due to the hormonal property of the material rather than to incidental disturbances of nutrition.

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THE HYPOPHYSIS AND HEMOPOIESIS[1]

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THAT HORMONAL CONTROL of the formation of cells of the blood may exist is a logical hypothesis. Certain clinical and experimental evidence (1–22), is suggestive, and some (9, 19) have gone so far as to conclude that the anterior pituitary body furnishes a hemopoietic hormone. Our interest in the possible relationship of the endocrine organs, the hypophysis in particular, to hemopoiesis has led to a series of experiments during the past few years. All these experiments have been carried out with rats and the technical procedures have been those commonly in use.

White rats, virtually all males from 50 to 100 days old and weighing from 125 to 200 gm. were used in all the studies. The surgical procedures were carried out under ether anesthesia, and hypophysectomy was performed by the parapharyngeal approach. All animals were fed on the Steenbock ration, to which glucose was added for the hypophysectomized animals. Complete counts were made twice a week upon peripheral blood taken from tail veins. Erythrocyte and leukocyte counts were made in duplicate, paired pipettes being used. Counting chambers and pipettes were standardized by the United States Bureau of Standards. Hemoglobin determinations were made with the Haden-Hausser hemoglobinometer, which was so standardized that 15.4 gm. of hemoglobin per 100 cc. represents 100%. Reticulocyte counts were made by examining 1000 cells on dried cover-slip smears stained with cresyl blue. In all the early experiments differential counts were made upon hypophysectomized animals, but since no significant alterations were demonstrated, these were omitted in the more recent studies. In other experiments differential counts were made where indicated. A routine postmortem examination of animals from which an organ had been extirpated was made at the conclusion of the experiments to determine whether the surgical removal had been complete. Sections of the trachea in the thyroidectomized rats were studied microscopically to make certain that no tissue remained in attachment.

The marrow studies here reported are based on the examination of material taken from the femurs. It may be well to describe the method for obtaining the marrow since many unsatisfactory methods were tried before the present very adequate procedure was developed. The entire femur was disarticulated on each side. With small heavy manicure scissors a slit the length of the bone was made, and then each end of the bone was cut off. The fixative, Zenker's solution with 5% acetic acid, could then reach all portions of the marrow. After fixation had been completed and the sections were to be made, the remaining bone about the cylinder of marrow could be readily peeled off. The marrow was always found to be well fixed. The tissues were then cut into longitudinal sections, which were very satisfactory for study of the entire femoral marrow.

The experiments reported to date (16, 17) demonstrated the following (*a*) hypophysectomy, when complete, was always attended by a decrease in reticulocytes from a mean level of about 3% to less than 1%, the average postoperative level of 0.2% being reached within a period of 8 or 9 days. (*b*) A slowly progressive anemia followed removal of the hypophysis after an immediate postoperative polycythemia; which was presumably due to hemoconcentration resulting from diuresis. (*c*) Subjection of hypophysectomized rats, for periods of days

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[1] This study has been aided in part by a grant from the Wisconsin Alumni Research Foundation.

and weeks, to a reduced pressure of 422 mm. Hg, equivalent oxygen tension 12% of an atmosphere which corresponds to an elevation of approximately 16,000 feet, was not attended by reticulocytosis nor by polycythemia, as was the case observed in normal controls. Furthermore, red cell hyperplasia of the bone marrow did not occur under these conditions, although it was marked in control animals. (*d*) Splenectomy previous to hypophysectomy did not modify the negative response to the stimulus of oxygen want in the reduced pressure chamber. (*e*) The subcutaneous administration, 3 times a week, of antuitrin growth hormone (Parke, Davis & Co.) in doses of 0.5 cc. (5 rat growth units) and 1 cc. (10 rat growth units) to rats which had been hypophysectomized was invariably attended by marked reticulocytosis, which in some instances rose as high as 15% from an average pre-injection level of 0.2%. It was accompanied by no increase in hemoglobin or erythrocytes, but rather by a decrease in the levels of both, in association with bone marrow hyperplasia. Injection of the same doses of antuitrin growth hormone to normal unoperated rats produced no changes in the normal levels of reticulocytes (3%), hemoglobin, or erythrocytes. (*f*) The parenteral administration of large doses of liver extract had no effect upon the reticulopenia of hypophysectomized rats. (*g*) Thyroidectomized rats and orchidectomized rats subjected to reduced pressures in the chamber responded in much the same way as normal rats with reticulocytosis, polycythemia, and bone marrow hyperplasia. (*h*) The administration of thyroxin in 0.04 mg. doses 3 times a week to a small group of hypophysectomized rats was attended in most of the animals by an increase in reticulocytes and bone marrow hyperplasia.

From these results it was concluded that "the hypophysis probably does have an effect upon erythropoiesis, but further studies will be necessary to establish whether or not the effect is specific or merely a non-specific effect associated with growth. It is our impression that the results are probably dependent upon a hormonal action, but proof is as yet wanting" (17). It is the purpose of the present communication to record more recent experiments. The procedures for handling the blood and animals were those previously reported (17) as described above. The same strain of male rats, most of them weighing from 140 to 200 gm., but a few as little as 125 gm. were used in these later experiments.

EXPERIMENTS

In the original studies, where hypophysectomized animals were given antuitrin growth hormone, an increase in reticulocytes resulted, but strangely no increase in hemoglobin and erythrocytes. These experiments, however, were pursued for only 5 weeks or less, and it was suggested that if the hormone had been administered for a longer period, erythrocyte and hemoglobin increments might have been observed. Hence the experiment was repeated with 4 hypophysectomized rats. Five-tenths of a cc. (5 U) of antuitrin growth hormone was administered subcutaneously 3 times a week for 9 weeks, at the end of which period the average erythrocyte count and hemoglobin were still below the control levels, despite the persistence of a reticulocyte level distinctly above the average pre-injection count of less than 0.1%. The results are shown in figure 1.

The possibility suggested itself that the reticulocyte-stimulating factor in the extract was the growth hormone itself rather than some specific hormone. Therefore the material was boiled in a water bath in the original 20 cc. containers for half an hour to destroy the growth factor. The pH of the boiled substance was 8.75, whereas that of unboiled material was 8.55. That the factor was destroyed is evidenced by the fact that growth ceased in the 8 hypophysectomized animals which received this material. The experiments recorded in figure 2 indicate that much, but not all, of the reticulocyte-stimulating factor was lacking. This furnished presumptive evidence that the growth factor was at least principally responsible for the production of reticulocytosis, but it is not conclusive, since the heat may have also destroyed an additional unknown hemopoietic factor.

It has already been observed that hypophysectomized rats subjected to conditions of oxygen deprivation in a reduced pressure chamber failed to develop reticulocytosis, polycythemia, or bone marrow hyperplasia, and that the administration of antuitrin growth hormone produced reticulocytosis and some bone marrow hyperplasia, but not polycythemia. An experiment was therefore devised to test the possibility that the combined stimulus of oxygen want and hormone administration produces not only reticulocytosis but also increases in the erythrocytes and hemoglobin. The results, depicted in figure 3, clearly demonstrate that whereas the administration of the hormone produced only an increase in the reticulocytosis, the animals became polycythemic also if they were placed in the reduced pressure chamber. The evidence thus obtained emphasizes the need for the hormone to produce the usual physiological response to oxygen want and further makes clear that the extract used has potency.

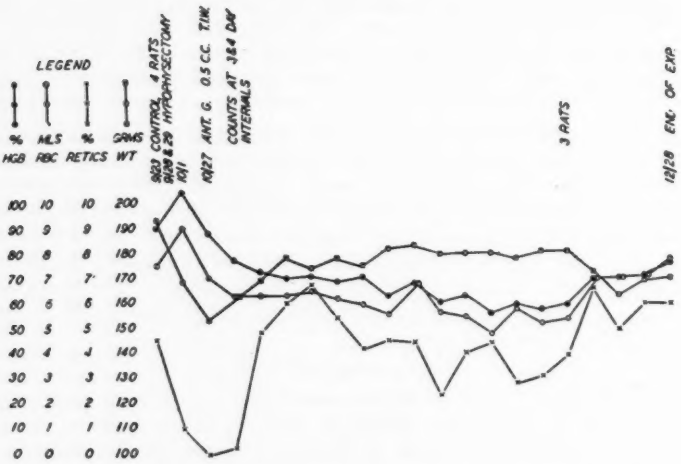

Fig. 1. The effect of administration of antuitrin growth hormone to hypophysectomized rats for 9 weeks.

It was suggested that the effect of hypophysectomy might be due to an indirect action through the thyroid. This possibility necessitated two groups of experiments. Each of 6 hypophysectomized rats was given 0.04 mg. of thyroxin intraperitoneally (sometimes subcutaneously) 3 times a week. The hormone was made up in an alkaline solution, of which 0.25 cc. furnished the desired quantity. The results, which were confirmed in other series of similar experiments, are shown in figure 4.

The sharp reticulocytosis that developed, similar to that induced by the pituitary hormone, subsided when the administration of thyroxin was stopped. It was not, however, accompanied by an increase in weight, as had been the case with the animals to which the growth hormone had been administered. The levels of erythrocytes and hemoglobin declined slowly. The bone marrow was moderately hyperplastic in all the rats, though less markedly so in some animals than in those given pituitary hormone.

Two rats in which the hypophysis was intact were given the same dosage of

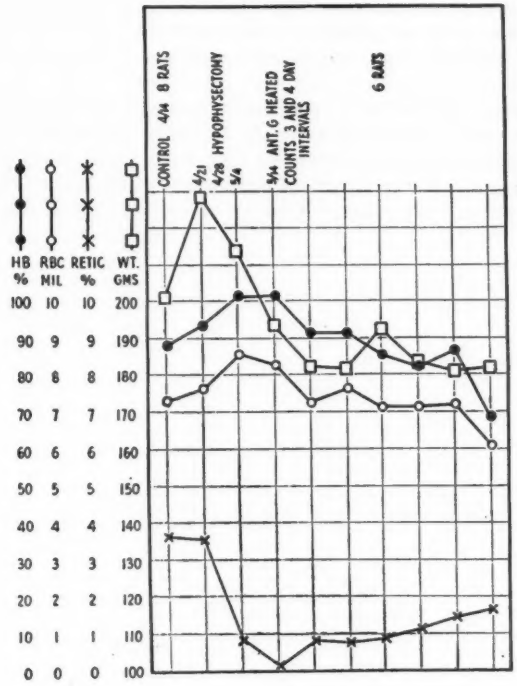

Fig. 2. The effect of administration of heated antuitrin growth hormone to hypophysectomized rats.

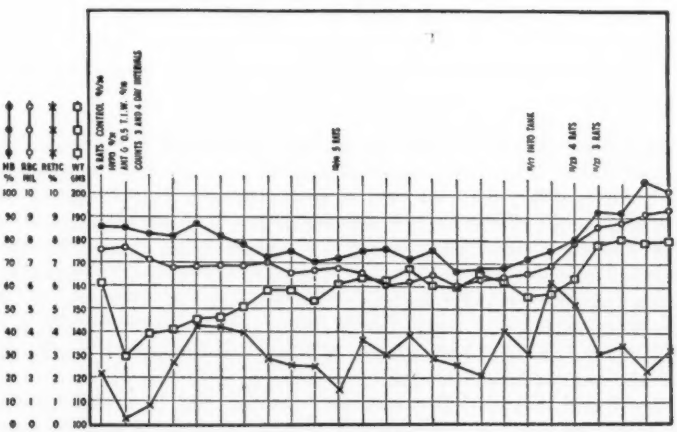

Fig. 3. The effects of combined antuitrin growth hormone and reduced pressure in a chamber upon hypophysectomized rats.

thyroxin, and these served as controls. In these animals, it will be noted, the control reticulocyte counts were fairly high, and they were not greatly modified by the administration of thyroxin. The weight curve indicates a progressive gain in these animals.

The next group of experiments were undertaken to demonstrate whether or not thyrotropic hormone (Ayerst, McKenna & Harrison) produced reticulocytosis in hypophysectomized rats. It was found that reticulocytosis did occur and the results are shown in figure 6. In the 10 normal rats used as controls a significant, but less marked reticulocytosis also resulted from the administration of this hormone. In

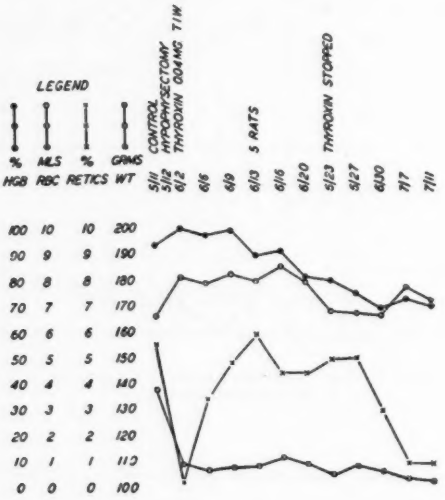

Fig. 4. The effect of administration of thyroxin to 5 hypophysectomized rats.

both the experimental and control groups the pre-injection levels of reticulocytes were reestablished when the administration of hormone ceased. The weight curves for the 2 groups differ; in the unoperated animals growth occurred during therapy, whereas the weight of the hypophysectomized rats was not significantly altered.

Next a group of rats were subjected to complete thyroidectomy. At first many of the animals developed tetany and died. Therefore the prophylactic administration of calcium and parathyroid extract became routine; each rat received 0.5 cc. of 10% calcium chloride and 0.5 cc. of parathyroid extract (50 U) immediately after operation and daily thereafter for at least a week, and then less frequent or smaller doses as long as they were under observation.

It was found that total thyroidectomy was followed by a slight decrease in reticulocytes, but this was less marked than in the case of the hypophysectomized rats. The results are shown in figure 7.

Four weeks after operation 1 cc. (10 U) of antuitrin growth hormone was ad-

ministered 3 times a week to the 9 of 12 rats that had survived the operation. The results showed slight, probably insignificant increases in the reticulocyte levels and in the weights of the rats. Then the injections were stopped and the rats were placed in the low-pressure chamber (422 mm. Hg); the response with respect to loss in weight, reticulocytosis, and increases in erythrocytes and hemoglobin was the same as that of normal animals.

Since thyroxin and thyrotropic hormone produced reticulocytosis in hypoph-

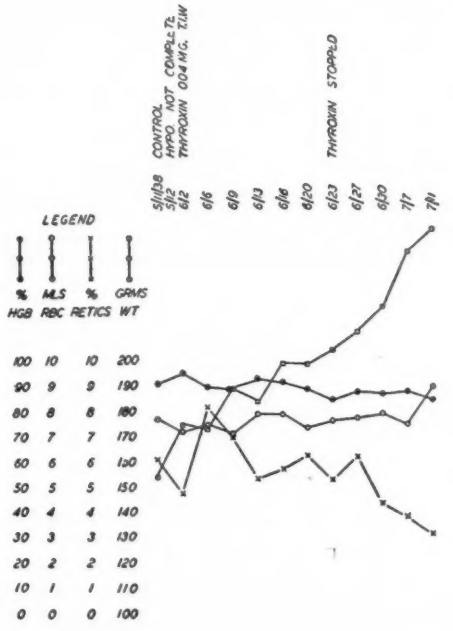

Fig. 5. The effect of administration of thyroxin to 2 normal rats.

ysectomized rats, similar to but not identical with that produced by antuitrin growth hormone (equivalent dosage of the reticulocyte-stimulating factor not determinable), it appeared that the responsible reticulocyte-stimulating factor in antuitrin growth hormone might be thyrotropic hormone material despite its deficiency,[2] or that the response to antuitrin growth hormone was non-specific. With this latter possibility in mind, 10 hypophysectomized rats were given subcutaneous injections of 0.5 cc. (5 U) of Armour's adrenotropic hormone (PH 3.5) 3 times a week. The results of this therapy are shown in figure 9.

[2] Dr. Oliver Kamm of Parke, Davis & Co., states, in a personal communication, that antuitrin growth hormone shows no gonadotropic activity when 20 mg. doses are administered to rats and no appreciable thyrotropic activity when 50 mg. doses are given to guinea pigs.

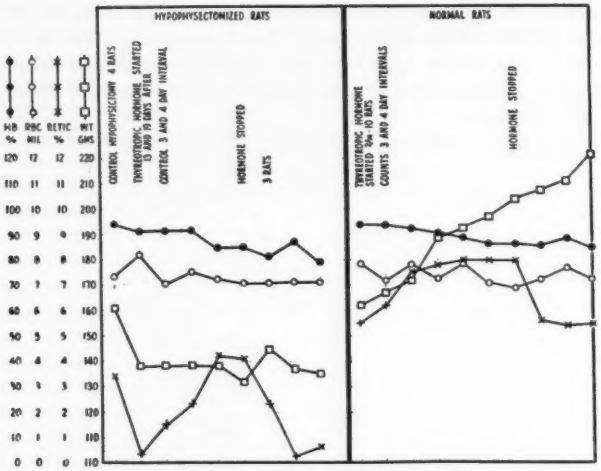
HYPOPHYSECTOMIZED RATS
NORMAL RATS
HB % RBC MIL RETIC % WT GMS
CONTROL HYPOPHYSECTOMY 4 RATS
THYREOTROPIC HORMONE STARTED 13 AND 19 DAYS AFTER
CONTROL 3 AND 4 DAY INTERVAL
HORMONE STOPPED
3 RATS
THYREOTROPIC HORMONE STARTED 3/4 - 10 RATS
COUNTS 3 AND 4 DAY INTERVALS
HORMONE STOPPED

Fig. 6. The effects of administration of thyrotropic hormone (0.5 cc. daily) to hypophysectomized rats and to normal rats.

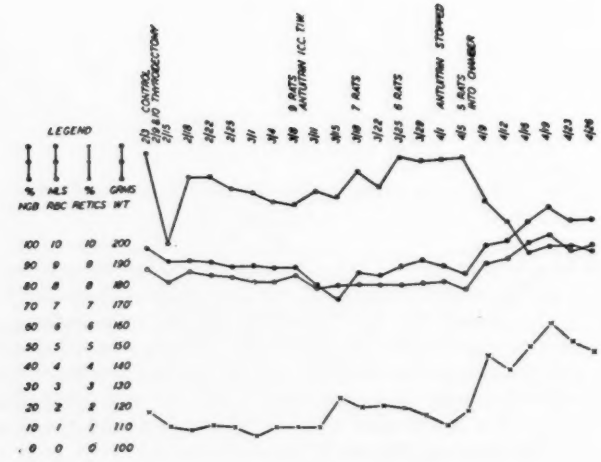

Fig. 7. The effect of administration of antuitrin growth hormone succeeded by reduced pressure in a chamber upon 10 thyroidectomized rats.

The resulting reticulocytosis, which persisted throughout the period of the injections and ceased when the administration of hormone was stopped, was as marked as one would have anticipated with the pituitary hormone. It will be noted that the usual post-hypophysectomy anemia persisted during the adrenotropic hormone therapy despite the persistence of a high reticulocyte count. These animals did not gain weight during the experiment, however, as did those that received growth hormone.

The results of these experiments raised the question whether or not the post-

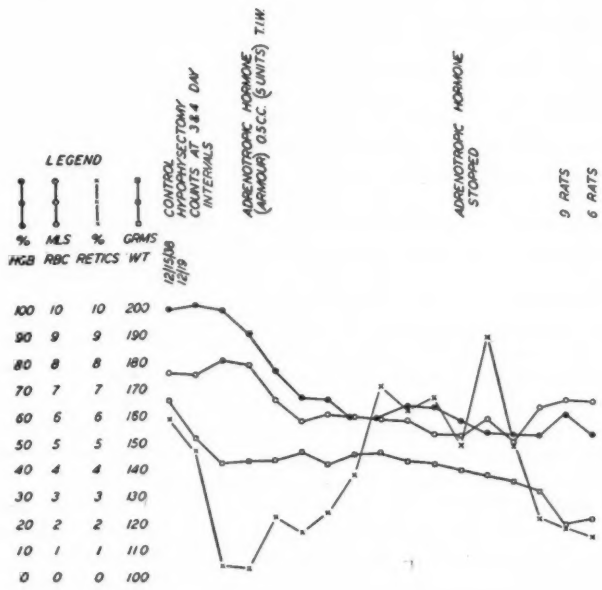

Fig. 8. The effect of administration of adrenotropic hormone to 10 hypophysectomized rats.

hypophysectomy reticulopenia and the reticulocytosis following the administration of growth hormone, thyrotropic hormone, and thyroxin might not be due to the effect of these substances upon the adrenals. Experiments wherein adrenalectomy was performed were not very successful and therefore inconclusive. Overbeek (18), however, has observed no reticulopenia following adrenalectomy. Dalton and Masson (23) observed a great increase in reticulocytes in the peripheral blood following adrenalectomy in rats.

A second possibility was next considered: that the reticulocytes in hypophysectomized rats were extremely labile, and that the responses to the injected materials were all non-specific and not due to the hormone content. The possibility that the pH of the solutions was a factor seemed unlikely, for boiled antuitrin growth hormone with a slight stimulating factor for reticulocytes was as alkaline, or more

so, than the alkaline unboiled material. Overbeek (18) maintains that the intraperitoneal administration of 20% glucose solution will produce reticulocytosis in hypophysectomized rats. We were unable to confirm this result upon repetition of his experiments in 3 rats. However, the subcutaneous injection of the sodium salt of cevitamic acid (Roche), pH 6.3 to 6.75, in 1 cc. (1000 international units) doses 3 times a week produced a reticulocytosis in 3 of 4 hypophysectomized rats. This response occurred between 10 and 14 days after the injections were started. The maximum count in one rat was 3.7%. The average post-hypophysectomy reticulocyte count in the 4 rats was 0.5%, and the highest average count for the group was 3.3%. Although this response figure is lower than that obtained with any of the hormones, it must be considered as significant. Boiling of the cevitamic acid for 30 minutes changed the pH to 6.85, whereas some of the unboiled material had a pH of 6.75. When the previously boiled material was administered, the reticulocytosis was even more marked. The average control level of the reticulocytes of this group was 0.4%, and after the injections the average maximal reticulocyte count was 7.2%. All the animals that received cevitamic acid became anemic and lost weight progressively during the experimental period. The explanation for the greater reticulocyte response following the injection of the boiled material is not at hand. It would seem, since both series of animals are small, that it might be accidental. The increase in the alkalinity of the solution is too slight to explain the discrepancy.

The daily subcutaneous administration of 5% solution of sodium bicarbonate to hypophysectomized rats, which produced local lesions after several injections, was also attended by significant reticulocytosis in each of 5 rats. The average pre-injection level of reticulocytes was 0.4%, and the average of the maximum counts was 5.1%. The counts were usually highest 2 to 3 weeks after the injections were started. All these rats were anemic at the conclusion of the experiments.

The administration of sodium bicarbonate and the same ascorbic acid to normal rats was followed in both instances by moderate increases in reticulocytes without associated anemia. In these animals the maximum reticulocyte counts occurred after one or two injections; they were not maintained at this level but continued to be higher than pre-injection levels as long as either substance was given.

These results obtained with sodium bicarbonate and cevitamic acid are not readily explained, but they indicate that the reticulocyte response in hypophysectomized rats is non-specific. That the alkalinity of the sodium bicarbonate solution is responsible scarcely seems logical, since boiled antuitrin growth hormone of similar alkalinity, and of greater alkalinity than the unboiled antuitrin growth hormone which produced marked reticulocytosis, has only slight reticulocyte-stimulating capacity. It is rather paradoxical that a non-specific stimulus such as an alkaline solution can produce marked reticulocytosis, whereas a specific stimulus to the bone marrow, such as oxygen want, does not.

That the lability of the reticulocytes in normal rats is great is suggested by Alt's (24) experiments. He showed that dietary deficiency alone would produce pronounced reticulopenia and that subsequent feeding would produce reticulocyte counts above the control level. He used young rats 33 days old, weighing about 50 gm. We have produced, in a group of rats with an average weight of approximately 150 gm., by 4 days of starvation, during which they were fed nothing but water, a reticulopenia as great as that following hypophysectomy. With the resumption of the Steenbock ration, marked reticulocytosis was observed, as high as 24.5% in one animal, after 3 or 4 days. The average height of the reticulocytes was similar to that attained by the administration of 1 cc. of antuitrin growth hormone to hypophysectomized rats. The results are shown in figure 9.

We were considerably puzzled in all our experiments over the production with antuitrin growth hormone of marked reticulocytosis in rats, which sometimes persisted as long as 9 weeks without an attendant increase in the hemoglobin or erythrocytes. In these cases the bone marrow was found to be hyperplastic, yet the blood level remained low. An obvious possibility was that there might be excessive hemolysis of cells. The rats never appeared to be icteric, but icterus might have been overlooked. Querido and Overbeek (20) found that the fragility of the red cells was not abnormal after hypophysectomy in rats. In one experiment 3 rats were given

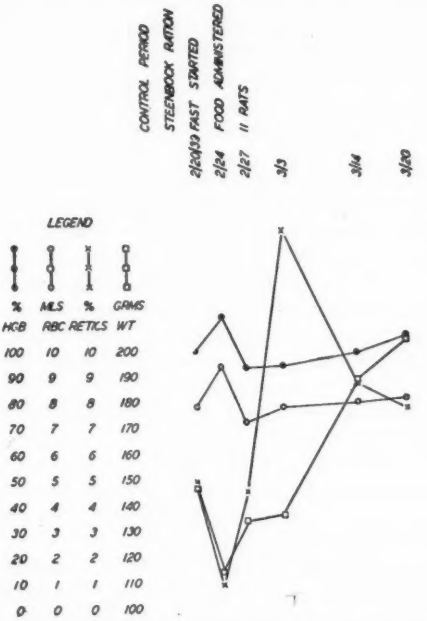

Fig. 9. The effects of fast and food administration in 12 normal rats.

antuitrin growth hormone (10 rat growth units) 3 times a week. Twice weekly, at the time the blood counts were made, the icterus index was measured. In no instance did any increase accompany the reticulocytosis; on the contrary, the maximum postoperative level of 15 U decreased to a minimum of 5 U at the termination of the experiments. Coincidentally, the usual reticulocytosis was evident. These results suggest that excessive hemolysis of red cells is not the explanation for the persistent or progressive anemia in the face of long-standing profound reticulocytosis.

DISCUSSION

Our previously reported experiments strongly suggested that the hypophysis did exercise a significant and possibly somewhat specific control over the bone marrow. This opinion was based on the marked changes which the peripheral blood underwent following hypophysectomy and the failure of these animals to respond as did

normal rats to the stimulus of reduced pressure in a chamber. Subsequent studies here reported have not tended to strengthen this early concept, however.

Since the results of the many experiments which have been, and are being, performed by others are not in entire agreement, these matters require some consideration at this time. Some facts are now available on which there has been practically complete agreement and these may also be stated.

Houssay, Royer and Orias (15) and Vollmer (22) and his associates, have reported that hypophysectomy is followed by anemia, and our findings are in agreement. Querido and Overbeek (20) have found that hypophysectomy does not affect significantly or consistently the total or differential leukocyte count. Vollmer (22) agrees, and our results in several hundred hypophysectomized rats are also in complete accord. Querido and Overbeek (20) have noted a decrease in the relative number of lymphocytes in the bone marrow, however.

Reticulopenia is an important result of hypophysectomy; in our experience with over 2 hundred rats it has invariably appeared within 10 days after hypophysectomy if the hypophysis has been completely removed. So far as we have been able to determine, although reticulocyte counts have not been made in all rats for weeks or months after operation, our data does not permit us to conclude that this reticulopenia always persists indefinitely. However, in many hypophysectomized rats observed for more than a month, the reticulopenia has always been persistent. But this does not mean that a reticulopenia is in itself a specific indication of disease or removal of the hypophysis. This matter is emphasized because there appears to be some misunderstanding regarding the significance which we attach to this finding. Reticulopenia may occur in normal rats for other reasons, as after starvation, but in only 7 of over 300 normal rats which we used in these experiments was the control reticulocyte count below 1%.

Querido and Overbeek (21) have found that hypophysectomized rats display a slight reticulocytosis following the administration of thyrotropic hormone. They are inclined to question its significance, stating that the influence on blood formation is totally different from that of the hypophyseal principle. Using a different thyrotropic material, we have noted that a pronounced reticulocytosis invariably follows administration of this hormone.

Flaks, Himmel, and Zotnik (9) have observed a reticulocytosis and an increase in hemoglobin and erythrocytes following the oral administration of fresh hypophyseal material to normal rats. They found too that injection of an acid fraction of a purified extract from which the growth hormone, gonadotropic hormone, and thyrotropic hormone had been removed by boiling at 100° for 5 minutes also produced polycythemia. They concluded that the hypophysis played an important physiologic rôle in the regulation and formation of the red elements of the blood by a hemopoietic hormone acting directly on the bone marrow and having no influence upon the thyroid. With injections of antuitrin growth hormone, deficient in thyrotropic and gonadotropic factors, we were unable to modify in any way the reticulocyte count or red cell and hemoglobin levels in normal rats. It may well be, of course, that the extract used by the Warsaw workers contained some substance besides the factor present in the material we used. Whereas Flaks et al. (9) found that the stimulating factor was heat-stable, Querido and Overbeek (21) observed that the active principle in their pituitary extract was heat-labile and was destroyed by boiling for 5 minutes. They found no reticulocyte-stimulating factor in growth hormone. (However, their growth hormone was purified by absorption on charcoal and elution with phenol, which possibly explains their negative results as the elution with phenol might have destroyed the active principle.)

In more recent experiments, Flaks, Himmel, and Zotnik (10) have reported that highly significant increases in the reticulocytes, hemoglobin, and erythrocytes, even to the point of polycythemia and of hyperplasia of the bone marrow, occurred in hypophysectomized rats following the oral administration of various extracts of the anterior pituitary containing the "hemopoietic" hormone. Their hypophysectomized rats given acetone extract of the hypophysis died with cachexia despite a normal blood count or even polycythemia. Never, with antuitrin growth hormone alone, have we been able to produce an increase in erythrocytes or hemoglobin, although reticulocytosis and bone marrow hyperplasia resulted. Because these workers inferred that the growth hormone we used contained a little of the hemopoietic hormone, the possibility that a more complete extract of the pituitary might affect the hemoglobin and erythrocytes was excluded when hypophysectomized rats were given the pituitary extract prepared by Squibb in 0.5 cc. doses parenterally thrice weekly. This extract contains thyrotropic and sex factors as well as the growth factor. The results were similar to those obtained with antuitrin growth hormone except that the reticulocytosis was less pronounced.

Polycythemia was produced in hypophysectomized rats subjected to the combined stimulus of reduced oxygen tension and the administration of antuitrin growth hormone. Whether the combination of thyrotropic or adrenotropic hormone and oxygen deprivation will produce polycythemia has not yet been determined. The fact, however, that the reduced pressure plus the hypophyseal hormone produced polycythemia, whereas neither alone was adequate, argues that the hormone we used must contain some of or probably most of the essentials in the extract of Flaks et al. (10). One would hardly regard the reduced oxygen tension as comparable to the so-called hemopoietic substance in the extract that they used. This is further emphasized by the fact that hypophysectomized rats develop no bone marrow hyperplasia when subjected to low oxygen tension alone.

Thyroidectomized and orchidectomized rats respond as do normal rats to reduced pressure. The removal of these endocrine organs, furthermore, does not produce a situation comparable to hypophysectomy so far as weight loss and the degree of reticulopenia are concerned.

The experiments involving the administration of thyroxin to hypophysectomized rats and the resultant reticulocytosis appear to be crucial. Here we are certain that the hormone is pure and does not originate per se in the hypophysis, yet it produces a response similar to that of the growth hormone so far as the hemopoietic organ is concerned. Querido and Overbeek (21) report in the body of their paper that most hypophysectomized rats to which thyroxin was administered died before reliable results could be obtained. In their conclusions, however, they state that injected thyroxin did prevent the post-hypophysectomy reticulopenia.

The reticulocyte response to both thyrotropic hormone and thyroxin suggests that the hypophysis may act through the thyroid to produce the blood changes, but the adrenotropic factor produces a similar reticulocytosis in hypophysectomized animals.

Querido and Overbeek (21) have found in their recent studies that hypophysectomized rats given injections of phenylhydrazine or bled by cardiac puncture respond as do normal rats with marked reticulocytosis. After injecting alkaline hypophyseal extracts into normal rats they found indications that blood destruction is increased. They conclude that the reticulopenia after hypophysectomy must therefore be due to diminished blood destruction. Previously (20) they had concluded that the reticulopenia occurring after hypophysectomy was evidence that the erythrocyte formation in the bone marrow had been altered. They found also (19) that

thyrotropic, gonadotropic, and growth hormones did not prevent the post-hypophysectomy reticulopenia and that the existence of a separate hormone, exerting influences on the blood picture, might be assumed. On the basis of our experiments it does not seem possible to agree with this assumption.

Our investigations led us to conclude that a specific hemopoietic hormone in the hypophysis acting directly upon the bone marrow probably does not exist, although this possibility has not been definitely excluded. The multiplicity of substances that will produce reticulocytosis in the hypophysectomized rat argues for this conclusion. Furthermore, we cannot fail to be impressed by the fact that starvation of normal rats will produce marked reticulopenia just as soon as, if not sooner than hypophysectomy; and that feeding after the period of starvation produces just as marked reticulocytosis as does the administration of growth hormone to hypophysectomized rats. These results emphasize the great lability of the reticulocytes in rats, whereas there is a comparative stability of hemoglobin and erythrocytes. The findings rather suggest that the alterations in the blood that occur after hypophysectomy may be due to general disturbances in metabolism attending the removal of this important organ rather than to a direct effect upon the marrow.

SUMMARY

The relationship of the hypophysis to hemopoiesis in rats has been investigated by removing various glands of internal secretion, the hypophysis in particular, and by administering various hormones and non-specific substances. The results have led to the conclusion that the hypophysis does affect hemopoiesis, but that this effect appears to be associated with general metabolic changes and probably is not dependent upon a specific hormone acting upon the bone marrow.

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AN EXPERIMENTAL STUDY OF THE SIMILARITIES BETWEEN CORTIN AND SOME CARDIAC GLUCOSIDES[1]

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A LARGE NUMBER of crystalline compounds have been isolated from crude extracts of the adrenal cortex. These have been identified as steroids in a series of exhaustive researches (1, 2, 3). They vary widely in their physiological activity, the exact sequence depending on the biological test used. The mother liquor or amorphous residue, after removal of these crystalline compounds, is believed to be from 10 to 100 times more potent than the best of the crystalline products (2, 4, 5). The last two authors also believe the more potent material has a greater number of hydroxyl groups than the corticosterone steroids.

From the same gland a strongly reducing sugar-acid was obtained (6), which has since been identified with ascorbic acid (vitamin C) (7).

Extraction of hormone activity from beef adrenal glands has been found possible with practically all aqueous and organic solvents with the possible exception of petrolic ether. This has been explained by alterations in solubility due to contaminating substances rather than to the wide solubility of the hormone. Steroid substances having adrenal cortical activity are quite insoluble in aqueous solvents and have no particular surface affinities. This last property we also feel is important since extracts having high potency can easily be made to form very stable emulsions. The high concentration of vitamin C in the adrenals and its morphological alterations in distribution lead us to speculate on the possible steroid-vitamin C linkage.

Evidence for such a combination would be (*a*) physiological activity of similar substances; (*b*) its isolation from adrenal glands and (*c*) enhancing the activity of cortically active steroids by linking them with ascorbic acid or a sugar. This article deals with *a; b* and *c* will follow.

On consulting Professor L. F. Fieser on the technic of isolating such a substance from the adrenal gland, it was suggested that we investigate the methods used in the chemical isolation of strophanthin, since it bore a close resemblance to our theoretical substance. A preliminary study of its physiological activity led us to believe that it might have cortin-like activity.

This point has been investigated in regard to protection against potassium poisoning, alteration of blood potassium levels and the level of serum protein. It has also been studied in regard to its ability to raise blood sugar and to protect normal animals against the convulsive action of insulin, which activities have previously been reported for adrenal cortex extract. The effects of repeated injections of this glucoside on the morphology of the adrenal cortex, were also observed. Other cardiac glucosides were also studied to see whether or not related substances also had like activity.

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Materials and methods. We have used normal and adrenalectomized cats, normal rats of the Long-Evans strain of hooded rats and the Rockland-Swiss strain of mice. Great care has been exercised in the maintenance of clean living conditions and uniform adequate diet of the animals. Operative and injection technic have been perfected to yield a minimum of trauma and excitement. The procedures used in the morphological studies and in the chemical extractions are best described in connection with the results obtained. This is also true of the detailed procedures in the individual physiological experiments.

The glucosides used were U. S. Reference Ouabain, Merck Strophanthin U.S.P. X, Pure Merck Digitalin German N. N. R. (p. 187) 1939, and Upjohn Digitora 1½ grain tablets. We are indebted to the Upjohn Company for these drugs and for a grant-in-aid of this research.

Effects on potassium. Zwemer and Sullivan (8) were the first to report that adrenal cortex extracts lowered blood potassium. This has been amply confirmed by other

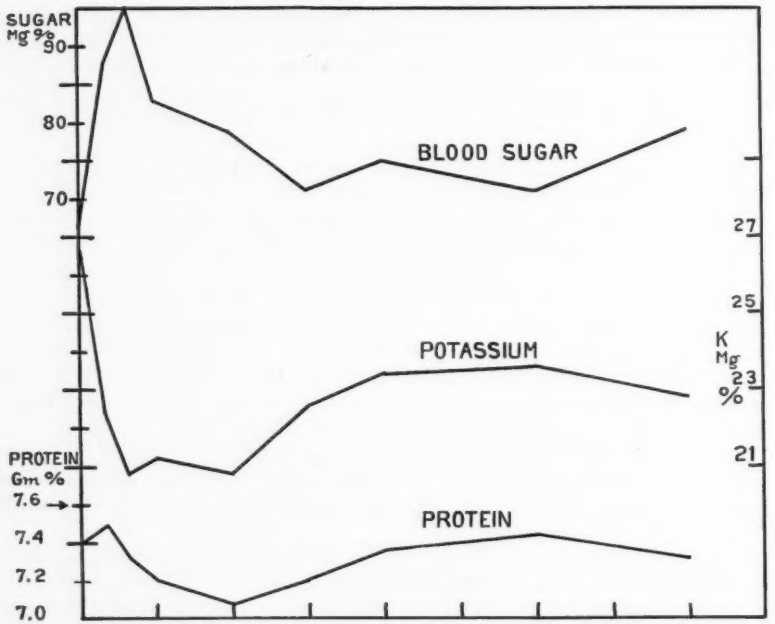

Fig. 1.

investigators. Since blood studies can best be followed as a curve, we made repeated determinations of the serum or plasma potassium level of normal cats after injections of strophanthin. These varied from sub-lethal amounts down to those giving no demonstrable clinical effects. In many of the cases, a significant decrease in the potassium level persisted for at least 18 hours after injection. It can be seen from the data that there were fluctuations in the level which are suggestive of two forces acting to maintain a constant. In previous work, we have found that fluctuations frequently appeared whenever an attempt was made to alter the plasma potassium level.

In addition to the effect on plasma potassium, we also observed an increase in the blood sugar and a decrease in the plasma protein concentration as estimated from the specific gravity. The elevation in sugar in one case is shown in the graph. All three effects have been reported to be obtained after injections of potent adrenal cortex extracts.

TABLE 1. EFFECT OF STROPHANTHIN ON BLOOD CONSTITUENTS OF THE CAT

Cat	0 Hr.	5 min.	10 min.	15 min.	30 min.	45 min.	1 Hr.	1 Hr. 30 min.	2 Hr.
				Plasma Potassium					
A	27.6	22.5	20.8	21.1	20.8	22.7	23.3	23.5	22.9
B	27.3	21.3	23.8	22.1		22.3	24.1	25.6	25.4
C	25.1	19.9	21.0	19.9		20.0	23.8	20.5	20.0
D	23.9	20.8	28.6	23.2	20.8	22.3	23.9	23.0	21.3
E	22.0	20.8	21.4	20.0		21.4	23.3	24.7	22.0
F	21.0	20.5	21.0	19.9	22.8		25.7	23.4	20.2
G	20.5	19.1	22.1	18.6		20.8	23.5	25.0	22.1
H	19.7	18.4	19.7	17.6	18.8		22.1		18.0
Av.	23.4	20.4	22.3	20.7	20.8	21.6	23.7	23.6	21.3
				Plasma Proteins					
D	7.93	7.93	7.69	7.66	7.32	7.31	7.31		7.34
E	7.58	7.35	6.54	6.75		6.71	6.02		6.47
G	7.50	7.41	7.30	7.56		7.30	7.17	6.95	7.07
A	7.41	7.49	7.34	7.17	7.07	7.13	7.37	7.41	7.31
F	7.21	7.09	7.21	6.64	6.64		6.69	6.02	6.10
B	7.06	7.08	7.02	6.93		6.69	6.92		6.64
C	7.06	7.02	6.93			6.64	6.74		6.60
H	6.78	6.51	6.37	6.23	6.23		7.20		6.85
I	6.21	6.14	6.25	6.12	6.50	5.92	5.57		5.32
Av.	7.19	7.11	6.96	6.88	6.75	6.81	6.77	6.76	6.63
				Blood Sugar					
E	111	124	124	128		124	92	92	92
F	100	120	135	145	131		131	98	101
A	71	87	96	83	79	71	75	71	79
C	61	90	85	90		96	90	83	80
B	58	83	79	83		83	92	79	75
G	50	75	75	83		87	81	67	75
Av.	75	97	99	102	105	92	93	80	84

Similar effects on potassium and blood sugar have been reported for the dog by P. Ginsburg (*Arch. f. Exper. Path. u. Pharm.* 128: 126, 1928).

The final low potassium level is perhaps due to three factors: blood dilution as shown by the fore-mentioned decrease in plasma protein content; renal elimination of potassium, which has been extensively studied in connection with cortical hormone and finally, cell fixation of potassium. This is compatible with the conception that potassium movements in and out of the cell are associated with its metabolic activity, potassium being lost when katabolism predominates and being gained by the cell when the anabolic processes are in ascendency.

Protection against lethal procedures. It has been our experience that cortical hormone will protect against a wide variety of toxic and lethal agents. Furthermore, we believe that a possible common denominator in these various conditions is an alteration in the differential permeability of the cells with a consequent release of potassium from the cells until it reaches lethal levels. We therefore, felt that protection against potassium itself would be a critical test for the potency of cortin. Our attempts to prove this point met with only indifferent success because we could not obtain a

sufficiently concentrated extract (9). Since that time Truszkowski and Duszynska (10) have found that after injection of lethal potassium, the percentage survival of mice varies directly with the dose of desoxycorticosterone. It is, therefore, significant to find that strophanthin and other cardiac glucosides will protect against otherwise lethal potassium injections and against otherwise convulsive insulin injections.

The data can best be presented in tabular form since we have varied both the protective doses of strophanthin, digitalin and oubain and the amounts of KCl given.

Lethal potassium. In a large series of experiments in this laboratory, it was found that 1 gm. of KCl per gm. body weight, was lethal for cats and mice when given intraperitoneally (9). For rats the lethal dose was 1.5 mg. per gm. body weight. With these amounts, convulsions appeared in about 1 hour or less and the animals died within 20 minutes of the onset of convulsions. This finding was confirmed for the present experiments on 10 cats, with no survivals; 10 rats, with no survivals and 32 mice, with 1 survival.

TABLE 2. CATS TREATED WITH BOTH STROPHANTHIN AND POTASSIUM CHLORIDE

Strophanthin	Elapsed time	KCl	Convulsed	Died
mg./kg.	*min.*	*gm./kg.*	*min.*	*min.*
0.15	90	1	110	140
0.15	90	1	150	
0.15	120	1		
0.15	120	1	150	
0.15	120	1	160	200
0.15	120	1		

Summary
6 animals, 4 survivals
2 animals at 90-minute interval, 1 survival
4 animals at 120-minute interval, 3 survivals

Lethal strophanthin. A series of experiments with strophanthin alone showed the lethal dose for cats to be 0.0002 mg. per gm., for rats 0.02 mg. per gm. and for mice, 0.0025 mg. per gm. It can thus be seen that mice are about 10 times as susceptible as rats, and cats are 100 times as susceptible as rats to strophanthin.

Protective doses. For protection against KCl, we gave the cats 0.0015 mg. per gm. which is 3/4 lethal; for the rats, we used 1/10 lethal for the protective dose, and for mice about 3/5 lethal. Of the 6 cats, there were 4 survivals; of the 38 rats, 29 survivals and of 138 mice, 109 survivals.

Potassium after strophanthin. The protection of normal animals against lethal amounts of potassium by previous treatment with a glucoside was successful with cats, rats and mice. Details of these experiments are given in tables 2 to 4. Four of six cats survived lethal potassium (table 2) although 2 of the survivors did have some difficulty 2½ hours after the potassium injection. Even extension of the survival period after lethal injections of potassium is significant. In previous work with adrenal cortex extract, we have never been wholly successful in protecting against lethal doses of potassium given to these animals.

The experiments on the rats are briefly summarized in Table 3. Best results were obtained with split doses of strophanthin beginning about 6 hours before the potassium. In the case of strophanthin in oil a single dose 18 hours before the injection of potassium was best. In working with mice it was possible to use larger groups of animals (table 4) and of 138 mice given lethal dose of 1 mg. of potassium chloride per gram body weight, there were 109 survivors. If one omits the 3-hour group, the percentage surviving after a single injection is 75. Of the survivors after the split dose

the percentage is 90. It can be concluded from these data that a previous injection of strophanthin is effective to a marked degree against lethal potassium. Conversely, in recent work on chronic administration of large amounts of desoxycorticosterone acetate, it has been found that the extremely low potassium and the symptoms of weakness which result, can be remedied by injection of potassium (12).

TABLE 3. RATS TREATED WITH BOTH STROPHANTHIN AND POTASSIUM CHLORIDE

Number	Strophanthin	Elapsed time	KCl	Convulsed	Died
		Split Dose			
1	2 γ/gm.	5, 3, 1 hours before	1.5 mg./gm.	85 minutes	120 minutes
2–8	2 γ/gm.	5, 3, 1 hours before	1.5 mg./gm.	none	none
9	2 γ/gm.	6, 4, 2 hours before	1.5 mg./gm.	70 minutes	80 minutes
10	2 γ/gm.	6, 4, 2 hours before	1.5 mg./gm.	65 minutes	100 minutes
11	2 γ/gm.	6, 4, 2 hours before	1.5 mg./gm.	70 minutes	
12	2 γ/gm.	6, 4, 2 hours before	1.5 mg./gm.	90 minutes	
12–16	2 γ/gm.	6, 4, 2 hours before	1.5 mg./gm.	none	none
		Single Dose			
17	2 γ/gm.	16 hours	1.5 mg./gm.	8 minutes	30 minutes
18	2 γ/gm.	16 hours	1.5 mg./gm.	45 minutes	60 minutes
19	2 γ/gm.	16 hours	1.5 mg./gm.	50 minutes	105 minutes
20	2 γ/gm.	18 hours	1.5 mg./gm.	150 minutes	overnight
21	2 γ/gm.	18 hours	1.5 mg./gm.	150 minutes	
22–27	2 γ/gm.	18 hours	1.5 mg./gm.	none	none
28	2 γ/gm.	24 hours	1.5 mg./gm.	40 minutes	45 minutes
29	2 γ/gm.	24 hours	1.5 mg./gm.	40 minutes	
30	2.25 γ/gm.	16 hours	1.5 gm./gm.	100 minutes	
31	2.25 γ/gm.	16 hours	1.5 mg./gm.		
32	2.25 γ/gm.	18 hours	1.5 mg./gm.		
33	2.25 γ/gm.	18 hours	1.5 mg./gm.		
34	2.25 γ/gm.	20 hours	1.5 mg./gm.	90 minutes	
35	2.25 γ/gm.	20 hours	1.5 mg./gm.		
36	2.5 γ/gm.	16 hours	1.5 mg./gm.	90 minutes	120 minutes
37	2.5 γ/gm.	16 hours	1.5 mg./gm.	55 minutes	
38	2 5 γ/gm.	16 hours	1.5 mg./gm.		

Summary

38 animals, 29 survivals
8 animals treated at 5, 3, 1 hours before with 2 mg./kg. strophanthin, 7 survivals
8 animals treated at 6, 4, 2 hours before with 2 mg./kg. strophanthin, 6 survivals
3 animals treated at 16 hours with 2 mg./kg. strophanthin, 0 survivals
8 animals treated at 18 hours with 2 mg./kg. strophanthin, 7 survivals
2 animals treated at 24 hours with 2 mg./kg. strophanthin, 1 survival
2 animals treated at 16 hours with 2.25 mg./kg. strophanthin, 1 survival
2 animals treated at 18 hours with 2.25 mg./kg. strophanthin, 2 survivals
2 animals treated at 20 hours with 2.25 mg./kg. strophanthin, 1 survival
3 animals treated at 16 hours with 2.5 mg./kg. strophanthin, 2 survivals

Protection against insulin. In determining a protective effect against insulin convulsions and death, we have endeavored to follow as closely as our laboratory permitted, the procedures used for the standardization of insulin. The mice were selected to be as nearly 20 gm. as possible and in most cases animals of differing weights were balanced in corresponding groups. The mice were fasted for 18 hours, then given an intraperitoneal injection of insulin and kept at a constant environmental temperature of 36 to 38° C. for a period of 2 hours. At this time most of the controls had convulsed and had either died or recovered. The aqueous solutions of strophanthin and other glucosides were administered in 3 doses at 6, 3 and 1 hours before the insulin injection. When strophanthin in oil was used, it was injected at the start of the fasting period. This gave an interval of 18 hours before insulin, which was found to be approximately optimal by preliminary experiment. The individual aniamals of a repre-

sentative experiment are given in table 5 and it can be seen that there is a definite protective effect of strophanthin and other glucosides against insulin, which, in some cases, is quite marked. A representative group of rats tested with strophanthin is given in table 6. A protective effect against insulin with adrenal cortex extract was found in rabbits by Zwemer and Hrubetz (12) and has recently been reported for mice by Jensen (13), and rats by Selye (14).

Effect of glucosides on adrenal cortex morphology. The similarity of action of the glucosides in relation to potassium and insulin with that of the effects of adrenal cortex extract, led us to study the adrenals of cats and rats treated with digitalis and strophanthin, in view of the report by Ingle (15) on adrenal cortex changes after large amounts of adrenal cortex extract or crystalline products.

TABLE 4. MICE TREATED WITH BOTH STROPHANTHIN AND POTASSIUM CHLORIDE

Number used		Strophanthin	Elapsed time	KCl	No. convulsed	No. died
			Split Dose			
A	6	2.0 γ/gm.	18 hours	1 mg./gm.	4	1
B	6	2.0 γ/gm.	20 hours	1 mg./gm.	2	2
C	6	1.5 γ/gm.	3 hours	1 mg./gm.	6	5
D	6	1.5 γ/gm.	17 hours	1 mg./gm.	4	3
E	6	1.5 γ/gm.	18 hours	1 mg./gm.	3	1
F	6	1.5 γ/gm.	19 hours	1 mg./gm.	2	1
G	24	1.5 γ/gm.	20 hours	1 mg./gm.	9	2
H	6	1.5 γ/gm.	22 hours	1 mg./gm.	3	2
I	6	1.5 γ/gm.	24 hours	1 mg./gm.	5	5
			Single Dose			
A	3	1.55 γ/gm.	6, 3, 2 hours	1 mg./gm.	1	0
B	3	1.55 γ/gm.	6, 3, 1 hours	1 mg./gm.	1	0
C	12	1.5 γ/gm.	6, 4, 2 hours	1 mg./gm.	5	3
D	12	1.5 γ/gm.	6, 3, 2 hours	1 mg./gm.	3	0
E	12	1.5 γ/gm.	6, 3, 1 hours	1 mg./gm.	2	1
F	18	1.5 γ/gm.	5, 3, 1 hours	1 mg./gm.	6	3
G	3	1.45 γ/gm.	6, 3, 2 hours	1 mg./gm.	0	0
H	3	1.45 γ/gm.	6, 3, 1 hours	1 mg./gm.	1	0

Summary: 138 animals, 109 survivals

After daily administration of ½ a digitora pill or 0.050 mg. strophanthin, the adrenals were removed and fixed immediately in neutral formalin. Frozen sections showed a definite decrease of lipoids in the cortical tissue. There were fewer fat-containing spongiocytes and there was a diminution in the inner zone cell size. When operated on, the animals were in good condition, and the structure of the gland indicated no toxic effects since the inner reticular zone and the sub-capsular zone showed no evidence of increased activity.

Preliminary chemical study. Beef adrenal glands obtained directly after killing were extracted with benzene, care being taken to prevent oxidation, hydrolysis or enzymatic action. After repeated distribution between aqueous and organic solvents and precipitation as a solid with subsequent recovery, we have obtained a substance that gives a positive Lieberman-Burchard test for steroid and a reduction of 2, 6, dichlorphenolindolphenol which suggests the presence of ascorbic acid. The material has been observed to crystallize out in microscopic amounts, but it is extremely labile and we have not yet obtained enough for structural analysis. Preliminary physiological tests have shown it to have a protective effect against potassium and insulin. The chemical study will be reported later in detail when quantitative tests have been made for each step.

Discussion. Experimental data have been presented which show that strophanthin and related glucosides bring about certain blood chemical changes and are effective in protection of normal animals against noxious agents in a manner similar to that of adrenal cortex hormone. These are summarized in table 7. A study of the morphologic alteration of the adrenal glands after glucosides, which is comparable to that obtained by Ingle (15) with adrenal cortex extracts and with crystalline products prepared by Kendall, continues this resemblance in activity.

The attempt to isolate our theoretical substance from the adrenal glands has been partially successful, but we have not obtained enough of the substance to adequately test its physiological activity; nor have we learned how to protect this labile compound so that its chemical structure can be analyzed.

TABLE 5. STROPHANTHIN PROTECTION AGAINST LETHAL INSULIN TO MICE

Number	Weight	Protection by	Insulin dose	Convulsed	Died
	gm.		u/kg.	time	time
RS 11	20	None	2.5	47	60
RS 12	24	None	2.5	50	50
RS 13	17	None	2.5	34	61
RS 14	20	None	2.5	20	28
RS 15	19	None	2.5	58	87
RS 1	20	None	2.0	55	60
RS 2	21	None	2.0	67	90
RS 3	22	None	2.0	80	
RS 4	18	None	2.0	50	75
RS 5	25	None	2.0	75	90
RS 6	20	None	2.0	41	82
RS 7	18	None	2.0	26	49
RS 8	19	None	2.0	70	
RS 9	21	None	2.0	49	78
RS 10	20	None	2.0	50	89
RS 84	18	None	2.0	50	79
RS 85	20	None	2.0	50	70
RS 66	20	None	2.0	50	72
RS 87	18	None	2.0	50	75
RS 88	21	None	2.0	50	80
RS 16	19	None	1.5	90	
RS 17	18	None	1.5		
RS 18	17	None	1.5	78	
RS 19	19	None	1.5	76	110
RS 20	16	None	1.5		
RS 21	19	2.5 γ/gm. of "S" in oil 18 hr. previously	2.0	57	98
RS 22	18		2.0		
RS 23	18		2.0	68	
RS 24	20		2.0	75	80
RS 25	21		2.0		
RS 26	20	2.0 γ/gm. of "S" in oil 18 hr. previously	2.0	65	71
RS 27	21		2.0	50	100
RS 28	17		2.0		
RS 29	18		2.0		
RS 30	19		2.0		
RS 31	20	1.5 γ/gm. of "S" in oil 18 hr. previously	2.0		
RS 32	25		2.0		
RS 33	23		2.0		
RS 34	22		2.0		
RS 35	19		2.0	47	85
RS 36	21		2.0		
RS 37	20		2.0		
RS 38	21		2.0		
RS 39	20		2.0		
RS 40	20		2.0		

TABLE 5.—*Continued*

Number	Weight	Protection by	Insulin dose	Convulsed	Died
	gm.		*u/kg.*	*time*	*time*
RS 41	25	1.5 γ per gram strophanthin in oil 22 hours previously	2.0		
RS 42	28		2.0		
RS 43	24		2.0	55	
RS 44	22		2.0	50	
RS 45	24		2.0		
RS 56	16	1.5 γ per gram oubain in water in doses 6, 3, 1 hour previously	2.0	53	
RS 57	17		2.0		
RS 58	18		2.0		
RS 59	20		2.0		
RS 60	17		2.0		
RS 61	19	2.5 γ per gram strophanthin in water in doses 6, 3, 1 hour previously	2.0		
RS 62	18		2.0		
RS 63	16		2.0		
RS 64	20		2.0		
RS 65	17		2.0	42	
RS 66	17		2.0		
RS 67	17		2.0		
RS 68	18		2.0		
RS 51	16	75 γ per gram digitalin in water in doses 6, 3, 1 hour previously	2.0		
RS 52	16		2.0		
RS 53	17		2.0		
RS 54	18				
RS 55	17		2.0		

TABLE 6. STROPHANTHIN PROTECTION AGAINST LETHAL INSULIN TO RATS

Number	Weight	Dose strophanthin per gm.	Interval	Dose insulin per kg.	Result
	gm.		*hr.*	u	
Protected					
19	212	10	21	15	survived
20	220	10	21	15	survived
21	200	10	21	15	survived
22	180	10	21	15	survived
23	180	10	21	15	CD
Control					
8	175	none	—	15	CD
11	245	none	—	15	CD
12	245	none	—	15	CD
13	260	none	—	15	CD
14	260	none	—	15	CD

This group of rats is the best combination of protection to convulsive dose. With higher doses of insulin the percentage protection decreases, and with lower doses more rats survived in both groups.

A critical test for the cortin-like activity of glucosides would be the maintenance, indefinitely and in good health, of adrenalectomized animals. In a preliminary report (16) we have suggested the possibility of prolongation of life after adrenalectomy provided one uses a large animal so that its condition may be repeatedly checked by blood studies. At that time we pointed out the difficulty of using strophanthin for maintenance since one is held between the narrow margins of toxic digitalization and adrenal insufficiency.

The strongest part of our evidence so far is the effect of strophanthin and other

TABLE 7. SIMILARITIES BETWEEN CORTIN AND SOME CARDIAC GLUCOSIDES

	Adrenal Cortical Hormone	Cardiac Glucoside
Lowers plasma potassium	x	x
Lowers plasma specific gravity (proteins)	x	x
Raises blood sugar	x	x
Favors polymorph. increase	x	x
Affects the electrocardiagram	x	x
Protection against lethal potassium	x	x
Protection against insulin death	x	x
Protection against diphtheria toxin	x	x

glucosides on the regulation of potassium by the body. This is especially true for the protection against lethal potassium. In our earlier experiments (9) we had great difficulty in protecting against potassium with the adrenal cortex preparations then available. Since that time it has been found possible to use the synthetic cortical hormone to protect against 50% lethal potassium and Truszkowski and Duszynska (10) report a linear relationship of percentage survival to the log dose of desoxycorticosterone acetate.

The resemblance of cardiac glucoside activity to that of cortical hormone receives indirect support from Dorfman's experiments using strophanthin on adrenalectomized mice (17). His operated animals withstood doses of 5 to 100 micrograms of strophanthin per gram body weight, for from 2 to 6 days. Our data on normal mice shows that they are unable to survive such doses for more than 15 minutes (table 8).

TABLE 8. STROPHANTHIN TO NORMAL MICE

Mouse	γ/gm.	Time to convulsions	Time to death
A	100	1 minute	2 minutes
B	50	3 minutes	4 minutes
C	25	6 minutes	7 minutes
D	10	7 minutes	12 minutes
E	5	4 minutes	13 minutes
F	3	(13 min. weak)	(30 min. recovering)

In the adrenalectomized animals an elevation in their plasma potassium may have counteracted the strophanthin as suggested by the experiments on daily injection of potassium chloride or strophanthin, or both, on rats (table 9). When both were given, the animals survived much longer than with either alone. This would suggest that both substances were acting antagonistically on the same mechanism.

This protective action has been tested against insulin since we felt that it might be a critical measure of cortin-like activity. The diabetogenic action of adrenal cortex preparations has been recently reviewed (18, a, b, & c) and it has been shown that potent cortical extracts will raise blood sugar and restore depleted glycogen in the liver and muscle of normal and adrenalectomized animals. These extracts also increase urine sugar in pancreatectomized animals that have been partially protected from diabetes by hypophysectomy or adrenalectomy. To some extent they prevent the fall in blood sugar after insulin (14) and its convulsive action on the nervous system (13).

Desoxycorticosterone and other cortin-like steroids isolated from the adrenal glands have been found to have a greater effect on sugar metabolism in the rat (18, 19) than in the dog (11) and in man (20). This difference may be associated with the

TABLE 9. ANIMALS TREATED WITH BOTH STROPHANTHIN AND POTASSIUM CHLORIDE

Daily strophanthin	Daily KCl	Days survived
mg./kg.	*gm./kg.*	
Chronic Rats		
	1.5	0
	1.5	0
	1.0	2
	1.0	2
	0.5	5
	0.5	7
	0.5	6
1.5	1.5	3
1.5	1.5	4
1.5	1.0	20
1.5	1.0	11
1.5	0.5	>20
1.5	0.5	>20
1.5		10
1.5		5
1.5		9

ability of the rat to synthesize vitamin C and thus with the synergistic action of ascorbic acid with corticoadrenal preparations.

The synergism of vitamin C with adrenal cortex has been shown in experiments attempting protection against lethal diphtheria toxin and more recently in work on asthenias of cortico adrenal origin. Protection against diphtheria toxin with digitalis was reported 8 years ago by Myers (21). The same author found that the polymorphonuclear leucocyte response to infection was much greater in guinea pigs previously injected with digitalis. This calls to mind the fall in the granuloctye count after adrenalectomy (22) and the increase in polys following injections of adrenal cortex extract found by Corey and Britton (23).

The cell sequence from capsule to reticularis in the adrenal cortex shows an accummulation of lipoids beginning in the sub-capsular zone and their disappearance in the fascicular zone (24). If this were due to a steroid combination with a water soluble side-chain making a polar molecule, an increase in this polar substance would tend to increase the total lipoid-aqueous surface area with the formation of smaller fat droplets until they became sub-microscopic in size. This would make the inner fascicular cells the region of maximal hormone concentration. The studies of Bourne (25) on vitamin C distribution in the adrenal cortex led him to postulate that it was in some way connected with the vital hormone. Its initial distribution between the lipoid droplets and its ultimate punctate appearance when visible fat drops are no longer present, are compatible with the possibility that the two may be combined.

Research on the chemistry of the adrenal cortical hormone may perhaps be a parallel of that on the cardiac glucosides where even now there is considerable disagreement as to the structure of the natural product found in plant cells. The difficulty there has been that enzymatic reactions which are involved in the synthesis of these complicated compounds may be reversed. The active cortin-like steroids so far obtained may have been isolated from gland extracts that had already been altered. This will remain a possibility until the chemical procedure is so controlled as to avoid enzymatic action, hydrolysis and oxidation from the moment the glands are removed from the animals in the slaughterhouse to the time of analysis of the finished product. These precautions may yield substances whose potency is as yet only a matter of speculation.

Conclusions. Evidence is presented to show that strophanthin when injected into

normal animals will lower the plasma potassium and protein levels and will raise the blood sugar. It will also protect normal animals against potassium poisoning and fatal insulin convulsions. These effects have previously been obtained with adrenal cortical hormone. Related cardiac glucosides are found to give these effects, but with quantitative differences. The possible nature of a more active adrenal cortex steroid than has heretofore been obtained is discussed. It is suggested that this is a steroid nucleus with a water-soluble side-chain, which may be ascorbic acid.

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THE EFFECTS OF SESAME OIL AND FRACTIONS OF SESAME OIL ON ADRENALECTOMIZED AND OTHER EXPERIMENTAL RATS

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SESAME OIL has been reported to be inactive when used in numerous experiments as a control oil or as a vehicle for fat-soluble substances. There are indications, however, that it may not be inactive under all conditions. Pollia (1) reported that when rats bearing sarcomas were injected intraperitoneally with sesame oil, there was marked damage to the viscera. Spurr and Kochakian (2) found that subcutaneous injections of sesame oil twice daily shortened the mean survival period of immature adrenalectomized male rats by 1.6 days as compared with untreated controls. They attributed this effect to the handling and treatment, noting that "handling and trauma are deleterious factors known to shorten the survival of adrenalectomized animals." Sandholzer (3) found that sesame oil was effective in prolonging the survival time of mice that were given a fatal injection of Friedländer's bacillus. Tobin (4) found that sesame oil denfitely prolonged the survival time of adrenalectomized pregnant rats that had successful gestation, and slightly enhanced their lactation.

To determine whether sesame oil was satisfactory as a control oil or as a vehicle for fat-soluble substances, its possible effects on normal and experimental rats were ascertained first. Later the effects of fractions of sesame oil were studied on adrenalectomized rats. For comparative purposes the effects of wheat germ oil, synthetic α-tocopherol,[1] synthetic tricaproin,[1] and pure linoleic acid[1] were studied on adrenalectomized rats.

All the animals used in these experiments were albino rats from our colony, originally of the Wistar strain. They were kept in uncrowded cages containing wood shavings, in a thermostatically regulated room at a temperature of 80° F. Purina Fox Chow, a commercial food, and tap water were supplied ad libitum. Most of the operations were performed under sodium amytal anesthesia. When needed this anesthesia was supplemented by drop ether. Only those animals which had recovered completely from the operation by the 2nd day are included in these data. Injections for the castrated animals were started on the day of operation, whereas injections for the adrenalectomized animals were started on the first postoperative day, unless otherwise indicated.

The Assay of Whole Sesame Oil on Normal and Experimental Rats

The effects of whole sesame oil on 119 normal, castrated, and adrenalectomized rats are summarized in table 1.

A. *Mature normal males.* Twenty-four males, were divided into 10 untreated controls, and 14 that were injected. The controls were observed for a period of 63 days.

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[1] The authors wish to thank Dr. J. B. Brown of Ohio State University and Dr. D. F. Robertson of Merck & Company, Inc., for generously supplying them with samples of pure linoleic acid and synthetic α-tocopherol, respectively. The tricaproin was obtained from the Eastman Kodak Company.

Four rats were injected subcutaneously with 0.05 cc. of sesame oil daily for 63 days. Subcutaneous oil cysts were found in these animals at autopsy. No definite toxic effects were noted, probably because of the small quantity of oil injected and the slow rate of absorption via the subcutaneous route.

Five rats of the group of 24 normal males were injected intraperitoneally with 0.25 cc. of sesame oil daily for 63 days. In these animals the body growth was slowly but progressively retarded until by the 63rd day they weighed only 80% as much as the controls (fig. 1) indicating that this quantity of oil was toxic to normal males. Further evidence of toxic effects was a 38% increase in adrenal weight as compared with the controls. The fact that the seminal vesicle weight decreased slightly indicates that the injected oil produced no androgenic stimulation.

TABLE 1. THE EFFECTS OF SESAME OIL ON NORMAL, CASTRATED, AND ADRENALECTOMIZED RATS

No. of rats	Initial body weight		Operative treatment	Sesame oil per animal		Effects
	Mean	(Range)		Daily[1]	Total	
	gm.			cc.	cc.	
10	131	(119–148)	none	—	—	
4	141	(134–148)	none	0.05 S	3.15	Survived until killed on 63rd day
5	130	(127–134)	none	0.25 I	15.75	Inhibited growth, body weight only 80% of controls, increased adrenal weight, decreased testis weight, slightly decreased seminal vesicle weight, after 63 days of injections
5	150	(144–157)	none	1.0 I	10 to 35	1 died on 10th, 17th, and 20th days, 2 survived 42 days until killed, variable effects on body weight
5	52	(46–60)	none	—	—	
5	47	(42–54)	none	0.50 I	3.5	No significant effects on body weight or viscera
5	49	(42–56)	castrated	—	—	Typical castration effects
5	50	(46–50)	castrated	0.50 I	3.5	Same as controls, no effects on viscera
5	44	(40–49)[2]	spayed	—	—	Typical castration effects
5	47	(44–52)[2]	spayed	0.50 I	14.25	Same as controls, no effects on viscera
36	57	(35–90)	adrenal-ectomized	—	—	Mean survival time 8.8(3–13) days, gained 12 gm., then lost 8 gm.
23	65	(43–88)	adrenal-ectomized	0.05 S		M.s.t. 9.2 (4–14) days, gained 4 gm. then lost 8 gm.
6	60	(54–68)	adrenal-ectomized	0.50 I		M.s.t. 8.3 (7–10) days, practically no gain in body weight

[1] "S" refers to subcutaneous injections; "I" refers to intraperitoneal injections. Injections of adrenalectomized animals were started on the 1st postoperative day, whereas those for castrated animals were started on day of operation.

[2] Females (all others were males).

Three of the five animals that were injected intraperitoneally with 1.0 cc. of sesame oil for 10 to 35 days died on the 10th, 17th and 20th days, whereas the remaining 2 survived until killed on the 42nd day. These last two rats gained 82 and 115 gm. during the experiment, while those 3 that died had first gained from 16 to 55 gm., and then had lost from 34 to 48 gm. before dying. In all instances oil was found in the peritoneal cavities, and in one rat (which died on the 20th day) the oil may have been responsible for peritonitis. Thus large doses of sesame oil over a period of several days when administered via the intraperitoneal route may be toxic to normal male rats.

B. Immature normal males. Ten immature males were divided into one group of 5 untreated controls, and another group of 5 which were injected intraperitoneally with 0.5 cc. of sesame oil daily for 7 days. No significant differences in daily body weight were noted. On the 7th day all animals were killed and autopsied. There was

accumulated oil in the peritoneal cavity of the injected animals. The viscera in all instances appeared normal.

C. Immature castrated males. Ten immature males were castrated, and 5 were injected intraperitoneally with 0.5 cc. of sesame oil daily for 7 days. There were no differences in daily body weight between the 2 groups. On the 7th day all animals were killed and autopsied. There was accumulated oil in the peritoneal cavity of each animal that had been injected. The viscera appeared normal. The average weight of the seminal vesicles in these animals dropped to one-half of the normal value, which indicated that the oil possessed no androgenic properties.

D. Immature spayed females. Ten immature female rats were spayed, and 5 of these were injected intraperitoneally with 0.25 cc. of sesame oil on the 1st day, and with 0.5 cc. of oil on 28 successive days. The animals were killed and autopsied on the 30th day. During this period the average body weight of each group showed a gain of more than 80 gm. There was very little mammary tissue in any of the animals. The vaginae had opened in a few of the rats, and all uteri were infantile. The oil possessed no estrogenic properties, according to these results.[2]

E. Immature adrenalectomized males. Sixty-five immature male rats were adrenalectomized. Thirty-six untreated controls had a mean survival of 8.8 (3 to 13) days. Twenty-three rats which were injected subcutaneously with 0.05 cc. of sesame oil daily had a mean survival of 9.2 (4 to 14) days. Sesame oil hardly altered the average survival time of these adrenalectomized rats. Six animals that were injected intraperitoneally with 0.5 cc. of sesame oil daily had a mean survival of 8.3 (7 to 10) days. This tenfold increase in dosage shortened the average survival period slightly.

The Composition of Sesame Oil and its Fractionation

Sesame oil is a semi-drying oil obtained from the seeds of *Sesamum indicum*, usually by cold expression. The physical and chemical constants for this oil have been published in the "Handbook of Chemistry and Physics" (5). The saponifiable matter of the oil consists of neutral fats or glycerides of saturated and unsaturated fatty acids. Jamieson and Baughman (6) found that the oil contained 36% linoleic acid as glycerides. The unsaponifiable matter varied from 0.9 to 1.7% of the oil, and sitosterol, sesamin and sesamolin have been isolated from the unsaponifiable matter. Adriani (7) obtained sesamin by crystallization from an ethyl alcohol solution of the unsaponifiable matter. Bertram, van der Steur, and Waterman (8) established the formula ($C_{18}H_{16}O_6$) for sesamin. Villavechia and Fabris (9) obtained a higher alcohol ($C_{25}H_{44}O_7$) by extracting the barium soap of sesame oil with ethyl alcohol. Klamroth (10) identified this compound as sitosterol. Villavechia and Fabris described the remaining unsaponifiable matter as a thick, non-crystallizable oil, free from nitrogen, and containing a substance giving the Baudouin reaction. Adriani recovered sesamolin, a glucosidic compound ($C_{20}H_{18}O_7$) which gave a positive Baudouin reaction. Boeseken, Coden and Kip (11) synthesized sesamol ($C_7H_6O_3$) and its β-glucoside from piponeral; the β-glucoside gave a positive Baudouin reaction, thus showing the mechanism of the reaction. Adriani obtained from hydrolysis of sesamolin, sesamol and another compound, samin ($C_{13}H_{14}O_5$), which failed to give the Baudouin reaction.

A. Extraction. Refluxing with either methyl alcohol or ethyl alcohol was found to be satisfactory for the extraction of some of the alcohol-soluble constituents of sesame oil. Some of the alcohol-soluble material could be extracted with 90% methyl alcohol at a temperature of 56° C. for 6 hours. A quantitative study of the differences in the extracted materials was not made.

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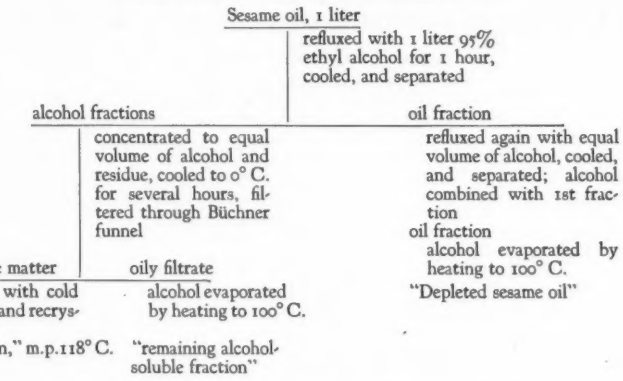
[2] Mr. Arthur L. Haskins assayed the sesame oil in immature rabbits by direct intrauterine application, and found that the oil possessed no progestational proliferative properties. This assay was repeated by Dr. Inés de Allende, who confirmed the negative finding of Haskins.

About 7 to 8 cc. of the alcohol-soluble material was obtained by refluxing a liter of 95% ethyl alcohol with a liter of sesame oil (table 2). After separating the oil

TABLE 2. ALCOHOLIC EXTRACTION OF SESAME OIL


Sesame oil, 1 liter
refluxed with 1 liter 95% ethyl alcohol for 1 hour, cooled, and separated
alcohol fractions
oil fraction
concentrated to equal volume of alcohol and residue, cooled to 0° C. for several hours, filtered through Büchner funnel
refluxed again with equal volume of alcohol, cooled, and separated; alcohol combined with 1st fraction
oil fraction
alcohol evaporated by heating to 100° C.
crystalline matter
oily filtrate
"Depleted sesame oil"
washed with cold alcohol, and recrystallized
"Sesamin," m.p. 118° C.
alcohol evaporated by heating to 100° C.
"remaining alcohol-soluble fraction"

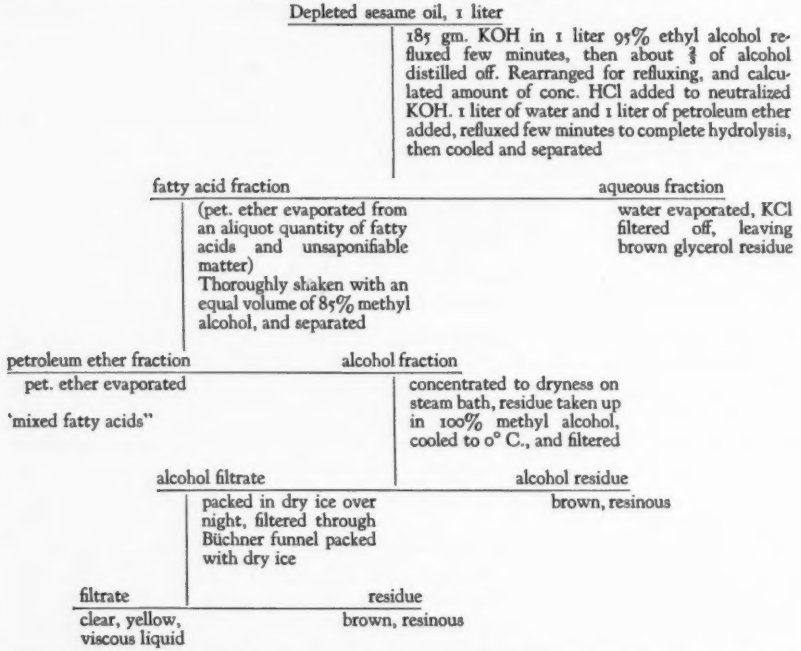
from the alcohol solution, the alcohol was distilled off at atmospheric pressure, leaving a brown liquid residue. By dissolving this residue in about 10 cc. of hot 95% ethyl alcohol and then cooling to 0° C. for at least 3 hours, about 200 mg. of crystals were obtained. The crystalline material had a melting point of 118° C., and was soluble in acetone, chloroform, pyridine, hot ethyl alcohol and acetic acid. It was insoluble in water, and it decomposed in cold concentrated sulfuric acid. These crystals, when dissolved in chloroform, produced a reddish color when 1 drop of concentrated sulfuric acid was added (Adriani's reaction); similarly in chloroform solution, a reddish blue color reaction was obtained when equal parts of concentrated sulfuric acid and acetic anhydride were added (Bömer and Winter's reaction). These color tests indicated the presence of sesamin. The result of the Baudouin reaction, which is specific for sesamolin, was negative. It was impossible to make either an acetate, benzoate, semi-carbizide, or phenylhydrazone derivative. Tocher (10), who extracted sesamin from sesame oil by means of glacial acetic acid, obtained a melting point of 118° C., and found the molecular weight to be 311 in benzene and 312 in acetic acid, making the empirical formula $C_{18}H_{18}O_5$. Although Villavechia and Fabris, Tocher, and Bömer and Winter (10) each assigned a different formula to this compound according to their own determinations of the composition and molecular weight, Bertram, van der Steur, and Waterman (8) established the formula $C_{18}H_{16}O_5$ for sesamin, with a molecular weight of 312, and a melting point of 122.7° C. Microscopic examination of the crystals obtained by crystallization from alcohol revealed two forms: colorless plates and needles. Repeated recrystallization yielded a few milligrams of plate-like crystals which had a melting point of 123° C. From these data the crystalline material was tentatively identified as sesamin.

The alcohol-soluble residue remaining after sesamin had been crystallized from the original alcohol-soluble mixture had the following characteristics. In the presence of 10 cc. of concentrated HCl and 0.1 cc. of recently distilled furfural dissolved in 10 cc. of 95% ethyl alcohol, about 1.0 cc. of this fraction developed a red color (Baudouin reaction). The alcohol-soluble residue when dissolved in chloroform in the presence of acetic anhydride and concentrated sulfuric acid developed a dark green color (Liebermann-Burchard test) indicating the presence of a steroid. Repeated attempts to isolate sitosterol in crystalline form from this residue failed. Any free fatty acids present in the sesame oil would be found in this fraction.

The oil that remained after alcoholic extraction was heated to 100° C. to evaporate a small amount of alcohol that was dissolved in it. This fraction was designated as depleted sesame oil.

B. Hydrolysis. Depleted sesame oil was first saponified and then the fatty acids were liberated by acidic hydrolysis, as indicated in table 3. Although the free fatty

TABLE 3. HYDROLYSIS OF DEPLETED SESAME OIL


Depleted sesame oil, 1 liter
185 gm. KOH in 1 liter 95% ethyl alcohol refluxed few minutes, then about ¾ of alcohol distilled off. Rearranged for refluxing, and calculated amount of conc. HCl added to neutralized KOH. 1 liter of water and 1 liter of petroleum ether added, refluxed few minutes to complete hydrolysis, then cooled and separated
fatty acid fraction
aqueous fraction
(pet. ether evaporated from an aliquot quantity of fatty acids and unsaponifiable matter)
Thoroughly shaken with an equal volume of 85% methyl alcohol, and separated
water evaporated, KCl filtered off, leaving brown glycerol residue
petroleum ether fraction
alcohol fraction
pet. ether evaporated
"mixed fatty acids"
concentrated to dryness on steam bath, residue taken up in 100% methyl alcohol, cooled to 0° C., and filtered
alcohol filtrate
alcohol residue
packed in dry ice over night, filtered through Büchner funnel packed with dry ice
brown, resinous
filtrate
residue
clear, yellow, viscous liquid
brown, resinous

acids are soluble in 100% methyl alcohol, they are not soluble in 85% methyl alcohol. The petroleum ether fraction, which contained both free fatty acids and any remaining unsaponifiable matter, was extracted in 250 cc. quantities with an equal volume of 85% methyl alcohol, and then separated. Evaporation of the petroleum ether fraction left a mixture of fatty acids, which at 20° C. separated into liquid and solid fractions. The methyl alcohol fraction which contained unsaponifiable matter was concentrated to dryness, and then dissolved in hot absolute methyl alcohol and cooled to 0° C., according to the method of Mackenzie, Mackenzie, and McCollum (12) for concentrating vitamin E in the unsaponifiable matter of wheat germ oil.

C. Comparison with effects of other lipid substances. With the exception of sesamin, found in the alcohol-soluble fraction of sesame oil, no fractionations were carried far enough to isolate and identify pure crystalline compounds. It was considered desirable, therefore, to compare the effects on adrenalectomized rats of other and related lipid substances with those obtained with sesame oil and its crude fractions. Wheat germ oil was chosen because of its high content of vitamin E. Since some beneficial effects were obtained with wheat germ oil, synthetic α-tocopherol was assayed in order to determine whether the vitamin E present in wheat germ oil was responsible for those effects. Sesame oil contains a large amount of linoleic acid as glycerides. Because linoleic acid is considered to be one of the essential fatty acids,

a pure sample of this acid was assayed. The iodine number, as determined by Dr. J. B. Brown, was 181, the same as the theoretical value. In order to ascertain whether neutral fats were responsible for the beneficial effects obtained in treated adrenalectomized lactating rats, synthetic tricaproin was chosen. We assume that this neutral fat, which is saturated and liquid at 20° C., is free from any contaminants with physiological activity.

The Assay of Fractions of Sesame Oil and Other Lipid Substances on Immature Adrenalectomized Rats

The resistance of an adrenalectomized animal to toxic substances is not as great as that of normal animals, due to the loss of adrenal cortical hormones. The survival time of an adrenalectomized rat, therefore, is a non-specific criterion for testing the toxicity of injected substances. Since the survival time of individual animals in a group varies, only the average survival time of the group may be used for purposes of comparison. When the average survival time of adrenalectomized immature rats is definitely shortened by the administration of the substance being assayed, that substance is considered to be toxic. Two hundred and sixteen immature male rats were adrenalectomized and used to assay fractions of sesame oil and other lipids (table 4). The average weight of these animals was 56 gm. A good correlation between the initial body weight and the survival time was not found. In assaying the fractions of sesame oil, a uniform daily dose of 0.05 cc., administered subcutaneously, was employed. For the crude alcohol-soluble fractions, this represented approximately 100-fold concentration, as compared with the original concentration in the oil. For the depleted sesame oil, the dose was practically equivalent to the original oil, there being only a very slight concentration.

Thirty-six uninjected adrenalectomized controls had a mean survival time of 8.8 (3 to 13) days.

The alcohol-soluble fractions of sesame oil, including crystalline sesamin dissolved in depleted sesame oil, shortened the survival period. When compared with the controls, the mean difference divided by the probable error of the mean difference for 25 animals so treated was 10.0. Thus there are present in sesame oil alcohol-soluble compounds, one of which is sesamin, that are toxic to adrenalectomized rats. It is suggested that the toxic effects of whole sesame oil upon normal male rats over a period of weeks may be attributed to the presence of these alcohol-soluble compounds.

Depleted sesame oil prolonged the average survival period of 29 rats. Since the individual survival periods ranged from 5 to 27 days, the significance of this enhanced survival is doubtful, although when compared with the controls, the ratio of the mean difference divided by its probable error was 4.0. The depleted sesame oil, however, was considered to contain some beneficial substances, as well as a slight amount of alcohol-soluble substances. A 10-fold increase in the daily dose of depleted sesame oil when administered intraperitoneally to 8 animals reduced the survival period to 7.2 days. Fractionation of the depleted sesame oil by filtration at 0° C. yielded 2 fractions one of which was solid at that temperature, and, when assayed, was almost as beneficial as the original mixture. When the depleted sesame oil, which was oxidized on the steam bath by bubbling oxygen through it for 25 minutes, was assayed on 17 animals, the survival time was 6.4 days. Hydrolysis of depleted sesame oil yielded crude aqueous, mixed fatty acid, and mixed unsaponifiable fractions. All these fractions shortened the average survival period. The inactivation of the beneficial substances in depleted sesame oil, consequently, may be the result of either oxidation or hydrolysis or a combination of both processes. The fact that an alcohol-soluble fraction was obtained after hydrolysis of the depleted sesame oil indicates

that alcoholic extraction does not remove all the alcohol-soluble constituents from sesame oil.

Wheat germ oil, synthetic α-tocopherol dissolved in depleted sesame oil, pure linoleic acid and synthetic tricaproin when assayed on immature adrenalectomized rats also shortened the average survival period (table 4).

TABLE 4. SURVIVAL TIME OF IMMATURE ADRENALECTOMIZED MALE RATS

Fraction assayed	No. of animals	Daily dose	Survival time	
			Average	Range
		cc.[1]	days	days
Controls	36	—	8.8	3–13
Alcohol-soluble				
Crude mixture	11	0.05 S	5.1	3–7
Sesamin[2]	9	0.05 S	5.1	3–10
Crude mixture minus sesamin	5	0.05 S	5.8	3–10
Depleted sesame oil	29	0.05 S	11.5	5–27
Depleted sesame oil	8	0.50 I	7.2	5–11
Depleted sesame oil, liquid at 0° C.	6	0.05 S	5.8	5–11
Depleted sesame oil, solid at 0° C.	10	0.05 S	10.1	6–14
Depleted sesame oil, oxidized	17	0.05 S	6.4	3–11
Fatty acids and unsaponifiable matter	6	0.05 S	3.8	2–7
Mixed fatty acids	7	0.05 S	5.7	5–7
Unsaponifiable matter	10	0.05 S	5.9	3–9
Glycerol and water-soluble matter	4	0.05 S	7.5	7–8
Wheat germ oil	11	0.05 S	5.9	3–9
α-tocopherol	8	0.50 mg.	7.3	5–11
α-tocopherol	7	0.05 mg.	7.1	5–9
α-tocopherol	8	1.0 mg.	6.2	4–8
Linoleic acid	5	0.03 I	less than 24 hours	
Linoleic acid	4	0.03 S	7.5	6–9
Tricaproin	15	0.05 S	5.7	4–9

[1] Subcutaneous injections are designated by "S"; intraperitoneal by "I." All injections were started on first postoperative day.

[2] 1 mg. sesamin/0.05 cc. of depleted sesame oil.

Although the adrenalectomized rat is more susceptible to histamine poisoning than the normal animal, the resistance to histamine can be raised by the administration of the adrenal cortical hormone (13). Immature male rats which weighed from 39 to 69 gm. were adrenalectomized, and were injected with histamine on the fourth postoperative day. The minimum lethal dose of histamine diphosphate for operated rats was 20 mg./100 gm. of body weight. Nine rats were injected subcutaneously with 0.05 cc. of sesame oil for 4 days, and all died from histamine poisoning within 58 to 232 minutes, at a dosage level of 10 mg./100 gm. Eight rats were injected subcutaneously with 0.05 cc. of depleted sesame oil for 4 days. They died within 59 to 377 minutes, at a dosage level of 20 mg./100 gm. Seven animals which were injected intraperitoneally with 0.15 cc. of depleted sesame oil died within 40 to 331 minutes when given the same dose as above. Five normal animals of the same weight range survived after a similar injection of histamine. Sesame oil and depleted sesame oil did not possess any cortin-like protective action against histamine poisoning.

The Effects of Depleted Sesame Oil and Other Lipid Substances on Adrenalectomized Lactating Rats

A. *Depleted sesame oil.* Rats adrenalectomized a few days before parturition will deliver their young, but will not lactate sufficiently to raise the young (14). Twelve primiparous females were adrenalectomized on the 16th to 19th day of gestation. They were then injected intraperitoneally with 0.25 cc. of the depleted sesame oil daily, beginning with the first postoperative day, and continuing for 8 to 30 days. Three females failed to wean any of their young, and 9 weaned from 14 to 100% of their suckling young. Three females survived for 81, 82 and 83 days and were killed.

TABLE 5. PRIMIPAROUS RATS ADRENALECTOMIZED 15TH TO 19TH DAY OF GESTATION

Group	No.	Daily dose	Average postoperative survival time	Young weaned
		cc.	days	%
Normal (unoperated)	8	0–0.5	—	92
Control[1] (operated)	10	—	9.6	0
Sesame oil[1]	15	0.15–0.55	21.0	0
Depleted sesame oil	19	0.1–0.5	33.0[2]	47
Wheat germ oil	9	0.1	26.6	34
α-tocopherol	9	2.5–10.0 (mg.)	26.0	—
Tricaproin[3]	13	0.05–0.10	32.4	31

[1] C. E. Tobin, in press, 1940.
[2] Not including 4 animals which had an indefinite survival time.
[3] Adrenalectomized just before, or just after parturition.

One of these 3 weaned 7 (58%) of her litter, another weaned 1 (14%) of her litter, and the third failed to wean any. At autopsy only the second female showed gross evidence of an adrenal rest. Lactation was not complete in the other 2 females, and there was no gross evidence of regenerated adrenal tissue. Consequently the cause of the prolonged postoperative survival could not be ascertained. There were 3 females, however, which weaned 100% of their young, exhibited incomplete lactation, and died of adrenal insufficiency on the 35th, 38th and 61st days. The young

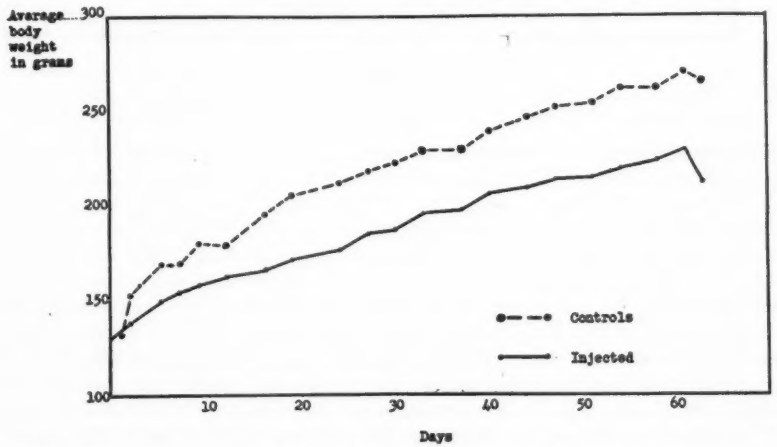


Fig. 1. THE EFFECT OF DAILY INTRAPERITONEAL INJECTIONS OF 0.25 CC. OF SESAME OIL ON THE AVERAGE BODY WEIGHT OF NORMAL MATURE MALE RATS.

were stunted during the period of suckling, but they gained rapidly as soon as they had been weaned (fig. 2).

For controls, 8 normal primiparous rats were selected, and 4 were injected intraperitoneally with 0.5 cc. of depleted sesame oil daily for 9 to 16 days during lactation. The oil was saturated with Sudan III in order to facilitate tracing its absorption. At autopsy stained fat was found in the peritoneal cavity, omentum, and hepatic lymphatics. No toxic effects were noted, and the suckling young gained weight at a normal rate. Examination of the milk in the stomachs of the suckling young showed unstained droplets of fat. The mesenteric fat of the suckling young was not colored.

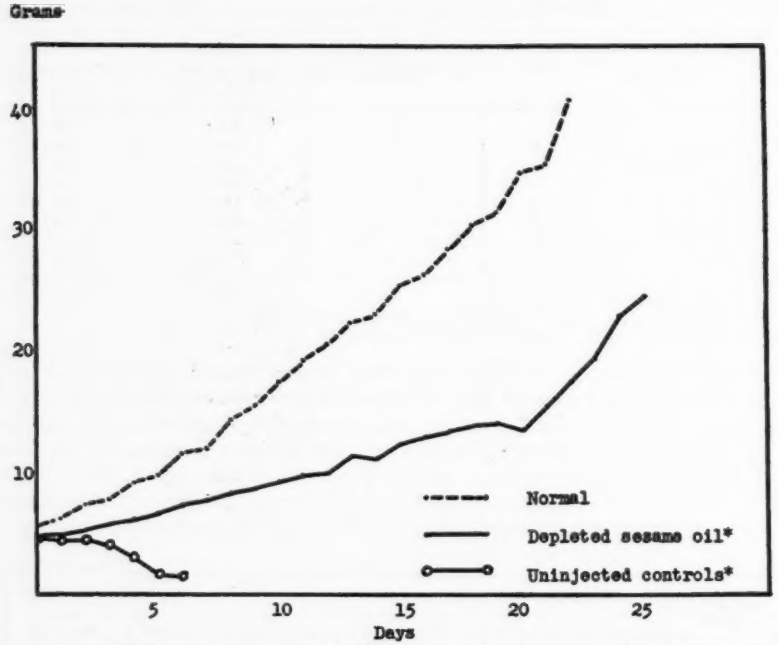


Fig. 2. Growth of suckling rats. *Lactating females were adrenalectomized before parturition, and were injected intraperitoneally, daily. All the young from the uninjected controls died within 1 week.

Seven primiparous rats were adrenalectomized on the 16th to 19th day of gestation. Beginning with the day of operation, they were injected intraperitoneally with 0.5 cc. of Sudan stained depleted sesame oil, daily for 5 to 22 days. All 7 delivered normal litters of from 7 to 13 each. Two failed to lactate adequately and lost all of their young, and the remaining 5 weaned from 60 to 100% of their young. One litter of these 5, however, survived for 21 days and the young were weaned, but they died within 2 days. All the young of the other 4 litters which had been stunted and weaned, survived and gained rapidly after being weaned. The adult females had a postoperative survival time ranging from 23 to 60 days, and all died of adrenal insufficiency. At autopsy there was stained fat in the peritoneal cavities, omenta, and hepatic lymphatics. Microscopic examination of the mammary glands and the renal fat deposits showed unstained fat in the cells. Smears of the peritoneal cavity, stained with methylene blue showed droplets of Sudan stained oil and many white cells. Droplets of fat within the cells were finely divided and gave no positive evidence of Sudan staining.

Seventy-six young rats (47%) were weaned successfully from 19 primiparous rats that were adrenalectomized before parturition and were treated with depleted sesame oil during lactation. The possibility of functional adrenal cortical tissue being responsible was eliminated by the fact that 15 of the females died of adrenal insufficiency. Furthermore, comparison of 2 rats, each 22 days old, that were from different litters, one nursed by a lactating female that had been subtotally adrenalectomized several weeks previously, the other nursed by a totally adrenalectomized lactating female maintained on depleted sesame oil showed a striking difference. The former weighed 45 gm. and appeared normal, whereas the latter weighed 20 gm. and was

Fig. 3. STUNTED SUCKLING RAT, 16 DAYS OLD, BORN OF A RAT ADRENALECTOMIZED BEFORE PARTURITION AND TREATED WITH 0.5 CC. SUDAN-STAINED DEPLETED SESAME OIL DAILY. Weight: 12 gm.; length: 67 mm.; tibial diaphysis: 9 mm. Notice extent of ossification of tarsal and metatarsal bones. About natural size. Fig. 4. NORMAL SUCKLING RAT, 10 DAYS OLD, BORN OF A NORMAL RAT THAT WAS INJECTED WITH 0.5 CC. SUDAN-STAINED DEPLETED SESAME OIL DAILY DURING THE PERIOD OF LACTATION. Weight: 17 gm.; length: 76 mm.; tibial diaphysis: 8.5 mm. Notice extent of ossification of tarsal and metatarsal bones. About natural size.

stunted. Several of the stunted rats from 4 to 28 days old were anesthetized and photographed with Roentgen rays. The length of the tibial diaphysis, and the number and extent of ossification of the carpal and tarsal bones as well as the metacarpal and metatarsal epiphyses were taken as indices of skeletal development. Characteristic of the stunted groups was a rat 16 days old that weighed 12 gm. and exhibited skeletal development that was approximately equivalent to a normal young rat 10 days old that weighed 17 gm. (fig. 3, 4). For further comparison, a normal suckling rat 16 days old weighed 28 gm., was 37% longer, and had a tibial diaphysis 33% longer than the stunted animal of the same age chronologically. After the young rats had been weaned, however, the skeletal development was accelerated so that by the 28th day it was comparable to that of a normal animal of that age. When approximately 10 weeks old. 10 females and 7 males were mated. All females became

pregnant within the next month and delivered normal litters of 6 to 8 young. Eight males and 10 females, with an average weight of 120 gm. and about 10 weeks old, were killed and autopsied. All phases of the estrous cycle were found in the vaginal smears at autopsy. The weights of the following organs were within the range for normal animals of a similar age: testes, seminal vesicles, adrenals, kidneys, livers, abdominal viscera, hearts and lungs.

The death of some of the suckling young, from females adrenalectomized before parturition, might be attributed either to some prenatal effect of adrenalectomy, or to some dietary deficiency. The following two protocols suggest that a dietary deficiency, manifested by incomplete lactation, was responsible.

Rat 13 was a normal primiparous animal that was adrenalectomized 5 days before parturition. She delivered a litter of 10 living young, weighing 45 gm. On the 1st, 20th, and 22nd days postpartum, 1 of her litter died, and 2 died on the 23rd day. On the 24th day one more was killed for histological examination of some of the tissues. The female was injected daily for 30 days, beginning with the 1st postoperative day, with 0.25 cc. of depleted sesame oil. On the 22nd postpartum day the adrenalectomized female was exchanged with a normal female which had delivered 2 days previously a litter of 9 young, weighing 51 gm. On the day of exchange, the 9 young weighed 59 gm. but all had died by the 6th day. Thus the foster mother which had been adrenalectomized was unable to lactate sufficiently to maintain a normal litter. Meanwhile the normal female was able to feed and maintain the 4 remaining young of the litter from the adrenalectomized female. During those 4 days, there was a gain of 5.9 gm. per animal, which was equivalent to the average gain per animal in the 17 days prior to the exchange of lactating females. When the 4 young were 28 days old, and together weighed 84 gm., they were weaned, and by the 30th day they weighed 112 gm. The adrenalectomized female died on the 40th postoperative day of adrenal insufficiency. Histological sections of skeletal and cardiac muscle and the kidney of one of the young rats appeared more like fetal than normal tissues.

Rat 80 was a normal primiparous animal that delivered a normal litter, and was adrenalectomized on the first postpartum day. She was then given another litter of 8 young rats of the same age as her own litter, and was injected intraperitoneally with 0.5 cc. of depleted sesame oil daily during the period of lactation. One of the young died on the 3rd day, but the remaining 7 survived and exhibited stunted development, up to the time that they were weaned. On the 20th postpartum day the average weight of the young was 14.7 gm. as compared with an average weight of 35 gm. for normal young of the same age. The lactating female showed a slight initial loss in weight, but gained 21 gm. by the 20th day.

The average gain in body weight during the period of lactation for 8 normal, 10 uninjected adrenalectomized and 19 adrenalectomized rats treated with depleted sesame oil, are shown in figure 5. It will be noted that the sesame oil injections not only prevented the loss of body weight shown by untreated adrenalectomized animals but permitted a more prolonged gain in weight than shown by the normal stock animals.

The following protocol shows that oxidized depleted sesame oil does not produce the same results as reported above, but is toxic.

Rat 81 was a primiparous animal that was adrenalectomized the day before parturition, and was given intraperitoneally 0.5 cc. of oil daily for 8 days. She delivered a litter of 10 young, which survived for 7 days, and then lost 22 gm. before dying on the 13th day. At autopsy there was oil in the peritoneal cavity, and there was considerable congestion of the lungs, liver, spleen and kidneys.

B. Wheat germ oil. Nine primparous females were adrenalectomized on the 17th to 19th day of gestation (table 5). They were injected intraperitoneally with 0.1 cc. of wheat germ oil daily, beginning with the first postoperative day and continuing for 3 to 30 successive days. Seven females delivered living young, with from 2 to 11 in a litter; 1 delivered 7 dead fetuses, and 1 suddenly lost considerable weight at term and probably delivered, although no young were found. Five of the 7 females weaned successfully from 1 to 7 (25 to 87.5%) young of their litters. All 9 females died of adrenal insufficiency in from 12 to 57 days after the operation. Wheat germ oil under these conditions was responsible for maintaining 27 young rats until they were weaned. This represented 34% of the young in all the litters. In all instances the young were stunted, but within a few days after weaning they gained rapidly.

C. *Synthetic α-tocopherol.* Eleven primiparous rats were adrenalectomized on the 15th to 19th day of gestation (table 5). Two controls received 0.1 cc. of depleted sesame oil injected subcutaneously on the first postoperative day. The remaining 9 were injected subcutaneously with 2.5 to 10.0 mg. of synthetic α-tocopherol that was dissolved in 0.10 to 0.45 cc. of depleted sesame oil. All females gave birth to living young, except one which delivered 12 dead fetuses with intact membranes.

Of the 9 females that were given α-tocopherol, only 2 weaned any of their young. These 2 weaned 80% and 89% of their litters, a total of 12 young rats. In each instance the young were stunted while suckling, but gained rapidly after being weaned.

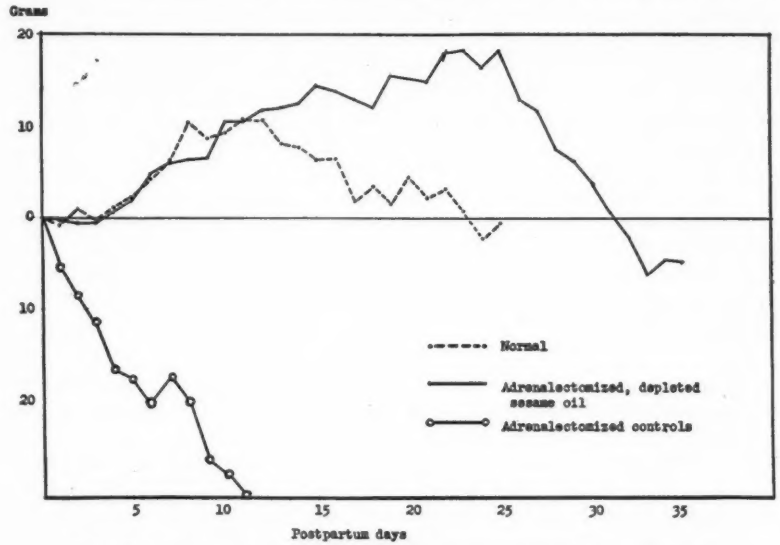


Fig. 5. THE EFFECTS OF ADRENALECTOMY AND DEPLETED SESAME OIL ON THE AVERAGE BODY WEIGHT OF LACTATING RATS.

Since one of the control animals, which was adrenalectomized before parturition, and was administered a total dose of 0.1 cc. of depleted sesame oil, weaned 75% of her litter of 8, and then died of adrenal insufficiency on the 46th day, it is considered that α-tocopherol did not benefit the 2 females which were treated with it. In view of the fact that 7 of the 9 females lost weight during lactation and failed to wean any of their litters, it is suggested that α-tocopherol is toxic to adrenalectomized rats during lactation. The α-tocopherol was also toxic to immature adrenalectomized male rats.

D. Tricaproin. Thirteen primiparous females were adrenalectomized either 2 to 4 days before or 1 day after parturition. They delivered normal litters of from 4 to 12 young each. They were injected intraperitoneally with 0.05 to 0.10 cc. daily of synthetic tricaproin, until the total dose ranged from 0.20 to 2.15 cc. Seven females lost all of their suckling young, and 6 weaned successfully 33 to 78% of their young. All the young were stunted, and those that were weaned successfully weighed on the average 14.5 gm. on the 20th day, as compared with 35 gm. for normal animals of the same age.

E. Linoleic acid. Six primiparous rats were adrenalectomized on the first or second day postpartum. They were injected subcutaneously with 0.05 to 0.10 cc. of linoleic acid daily, until the total dose ranged from 0.25 to 0.55 cc. In all, 33 young rats were

born, and only 1 survived the period of suckling and was weaned. Three others survived for 21 days. All 4 young rats were stunted. The acid formed palpable subcutaneous cysts, and was absorbed rather slowly.

The Effects of Administering Depleted Sesame Oil to the Suckling Young of Lactating Rats That Were Adrenalectomized before Parturition

Two normal primiparous rats were adrenalectomized on the 17th to 19th day of gestation. Each delivered a normal litter of 8 young rats that had an average weight of 5.1 gm. Beginning with the first day, each young rat received a total of 7 to 8 subcutaneous injections of 0.05 cc. of Sudan stained depleted sesame oil. The injections were administered about every 2nd or 3rd day, and the total quantity that any one rat received did not exceed 0.4 cc. The adult females received no injections. Thirteen of the original 16 (81%) exhibited markedly retarded gains in body weight while suckling, but survived after being weaned. On the 20th day the young rats had an average weight of 12 gm., as compared with 35 gm. for normal controls of the same age. By the 27th day, after they had been weaned, they had an average weight of 21 gm. At this time 8 were killed, and at autopsy an average of 0.1 cc. (0.05–0.20) of Sudan stained depleted sesame oil was aspirated from subcutaneous cysts. Thus the average net volume of injected and absorbed oil was about 0.25 to 0.30 cc. The adults during lactation lost 13 gm. on the 3rd day, slowly gained 19 gm., and then lost 31 and 64 gm., when they died of adrenal insufficiency on the 35th and 40th postoperative days.

DISCUSSION

Sesame oil, when injected into the rat, may be either inactive or toxic, according to the experimental conditions. Over a period of 9 weeks, daily intraperitoneal injections of 0.25 cc. of sesame oil produced toxic manifestations in normal mature males, and larger doses were sufficiently toxic to induce the death of the animal. Over a period of a few days, however, sesame oil, when assayed on immature rats, possessed neither androgenic, estrogenic, nor cortin-like properties.

The assays on the immature adrenalectomized male rats demonstrated the toxicity of the alcohol-soluble fractions of sesame oil and the beneficial effects of the depleted sesame oil (alcohol-insoluble fraction). In the alcohol-soluble fraction, both crystalline sesamin and remaining liquid residue were toxic.

Wheat germ oil (which is an excellent source of vitamin E), synthetic α-tocopherol, synthetic tricaproin, and pure linoleic acid, an essential fatty acid, failed to benefit immature adrenalectomized males. Neither oxidized depleted sesame oil nor the concentrated aqueous, the fatty acid, and the unsaponifiable fractions obtained from saponification of depleted sesame oil were beneficial when assayed with immature adrenalectomized male rats.

The depleted sesame oil consisted largely of glycerides of saturated and unsaturated fatty acids. This fraction not only prolonged the survival time of immature adrenalectomized rats as compared with untreated controls, but enabled female rats adrenalectomized before parturition to gain weight during lactation and to exhibit markedly improved lactation. In 4 litters 100% of the young survived beyond weaning, and in all litters of the group 47% of the young survived beyond weaning. Similar adrenalectomized females treated with wheat germ oil or with synthetic tricaproin lactated sufficiently to enable some of the young to survive beyond weaning. In each instance the suckling young were stunted, but they gained weight rapidly after being weaned, indicating, possibly, that there was a deficiency in the milk. The successful weaning of the litters could not be attributed to the presence of functional

adrenal cortical tissue, for most of the animals considered died of adrenal insufficiency after their young had died or had been weaned. The possibility of vitamin E was considered, for Karrer and Keller (15) reported finding 0.63% α-tocopherol in the unsaponifiable matter of sesame oil. Mason (16) found that depleted sesame oil was devoid of any vitamin E by bioassay with rats. Thus any tocopherol present in the oil was probably extracted by the alcohol.

Wang (17) found that the food intake in the rat increased enormously after parturition, and declined to the normal level after weaning. Sinclair (18) demonstrated that there was a change in the composition but not the amount of phospholipid fatty acids in the intestinal mucosa of rats. From this he suggested that phospholipids were an intermediate step in the resynthesis of neutral fat. At term the plasma neutral fat is about twice the non-pregnant level, and this lipemia does not disappear until late in lactation. Since the composition of milk fat changes relatively little, the quantity of milk is related to the amount of plasma lipids (19). Verzar and Laszt (20) found that adrenalectomized rats showed an impaired absorption of fatty acids by the intestinal mucosa, that was related to disturbances in the phosphorylating mechanism. Administration of the adrenal cortical hormone was sufficient to restore phosphorylation to normal. Barnes, Wick, Miller and MacKay (21) reported that adrenalectomized rats maintained on a 1% NaCl regimen absorbed neutral fats and methyl esters of fatty acids as readily as normal controls. They noted that the salt prevented the more severe symptoms of adrenal insufficiency, and thus the addition of salt evidently permitted fat absorption. Gaunt and Tobin (22), studying the effects of adrenalectomy during pregnancy and lactation, found they could control the percentage of young surviving until weaning by the amount of cortical extract administered. At least twice the life-maintaining dose was necessary for adequate lactation, and although lactation was improved, the young often exhibited sub-normal growth. Tobin (4) reported that sesame oil enhanced lactation of adrenalectomized rats, but none of the young survived until weaning.

Adrenalectomized animals are less resistant to toxins and infections than are normal animals, because of the lack of adrenal cortical hormone. Depleted sesame oil, from which a considerable amount of toxic substances were removed by alcoholic extraction, proved to be more beneficial to adrenalectomized rats than whole sesame oil. It is suggested that the high content of neutral fats of depleted sesame oil is responsible for the prolonged survival of immature adrenalectomized males, and for the improved lactation of females adrenalectomized before parturition. Intraperitoneal injection of neutral fat overcomes the inability of the intestinal mucosa to absorb sufficient fatty acids for the resynthesis of neutral fats to meet the requirements of lactation. Progesterone will substitute for the adrenal cortex in maintaining life in the adrenalectomized rat (23). Although immature male rats have no known source of progesterone, lactating rats are supplied with progesterone by the corpora lutea of lactation. Since injection of neutral fat into adrenalectomized and lactating rats keeps the young alive and nursing, involution of the mammary gland is prevented. After the young are weaned and the mammary gland atrophies, the mammary-anterior pituitary-ovary relationships are altered. With an apparent cessation of secretion of progesterone, the symptoms of adrenal insufficiency appear in a few days, culminating in the death of the animal.

SUMMARY

Sesame oil, when administered intraperitoneally in daily doses of 0.25 to 1.0 cc. for a period of several weeks, produces toxic effects in normal male rats. Extraction of sesame oil with ethyl alcohol yielded alcohol-soluble fractions, and an alcohol-

insoluble fraction, designated as depleted sesame oil. The alcohol-soluble fractions were toxic to immature adrenalectomized male rats, as determined by shortened mean survival periods. The depleted sesame oil prolonged the survival time of immature adrenalectomized males. Oxidized depleted sesame oil, all fractions resulting from hydrolysis of sesame oil, as well as synthetic tricaproin, synthetic α-tocopherol, wheat germ oil, and pure linoleic acid shortened the average survival period of immature adrenalectomized rats.

The survival time of lactating rats that were adrenalectomized before parturition was prolonged by depleted sesame oil, and lactation was improved markedly to enable 47% of the young to survive and be weaned successfully. All the young were stunted while suckling, they gained weight rapidly after being weaned, and when mature were mated and found to be fertile. Since similar results were obtained with wheat germ oil and synthetic tricaproin, but not with synthetic α-tocopherol dissolved in depleted sesame oil, the beneficial effects are attributed to the neutral fats. It is suggested that the injected neutral fats benefit the suckling young of adrenalectomized lactating rats, and since the young are maintained and suckle, mammary involution is prevented. This in turn by relationships with the anterior pituitary and ovary possibly influences corpora lutea of lactation to secrete enough progesterone to maintain temporarily the life of the adult female rat.

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THE EFFECT OF THE ADRENAL CORTEX ON CARBOHYDRATE METABOLISM[1]

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THE ROLE of the adrenal cortex in promoting the formation of carbohydrate from protein sources has been demonstrated in experimental animals (1). Recently it has also been shown that adrenal cortical hormone therapy had a striking effect in regulating carbohydrate utilization as well as in facilitating gluconeogenesis in patients with Addison's disease (2). The present study was undertaken to investigate the relationship between the increase in carbohydrate utilization and the decrease in gluconeogenesis which has been observed in adrenal cortical insufficiency. It appeared possible that an inability to form glucose from intermediate products of carbohydrate catabolism as well as from protein catabolism could account for many of the abnormalities.

In the present report the ability of adrenalectomized animals (phlorrhizin or insulin treated) to form glucose following the administration of potentially glycogenic substances has been studied. The comparative effectiveness of treatment with several adrenal cortical hormone preparations has also been observed.

METHODS

Male dogs (approximately 15 kg. in weight) were kept in metabolism cages and fed a constant diet composed of 350 to 400 gm. of ground beef to which was added 50 cc. of irradiated evaporated milk.[5] Bilaterally adrenalectomized dogs were maintained in good condition with subcutaneously implanted pellets of desoxycorticosterone acetate[6] (3). Blood glucose was determined on venous blood specimens by the Folin and Malmros micro method (4). In the insulin tests 0.25 U of insulin per kg. was injected intravenously. In the epinephrin tests 0.25 mg. per kg. of epinephrin was injected subcutaneously. Blood glucose determinations were made during the fasting state and at appropriate intervals following the administration of insulin and epinephrin. In the combined insulin and epinephrin test the epinephrin was administered 2 hours before insulin. A combined insulin and carbohydrate test was carried out by administering intravenously 250 cc. of a carbohydrate solution to be tested, the rate of administration being so adjusted as to be completed in a period of 45 minutes. Insulin was injected intravenously 15 minutes after the intravenous infusion of carbohydrate solution had been begun. Phlorrhizination was accomplished by injecting intravenously a

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[4] John H. Harris Fellow in Medicine.

[5] The milk used in these studies was supplied by the Irradiated Evaporated Milk Institute.

[6] The pellets of crystalline desoxycorticosterone acetate and the solution of desoxycorticosterone acetate in oil (percorten) were provided through the courtesy of Doctor E. Oppenheimer, Ciba Pharmaceutical Products, Inc., Summit, N. J.

fresh solution of 10% phlorrhizin (C. P. Eimer & Amend) in 5% sodium carbonate. This was supplemented by subcutaneous injections of 10% pulverized phlorrhizin in olive oil. Supplementary adrenal cortical extract[7] and desoxycorticosterone acetate therapy were given both subcutaneously and intravenously (a special aqueous preparation of desoxycorticosterone acetate being used intravenously). Seventeen-hydroxy-11-dehydrocorticosterone (compound *E*, Kendall)[8] and corticosterone in sesame oil were injected intramuscularly.

Male rats of the Wistar strain were kept in metabolism cages and were fed Purina dog mixture supplemented with vegetables. Adrenalectomized rats were maintained by means of 1% sodium chloride solution. Experiments were begun 1 to 2 weeks after bilateral adrenalectomy. Normal rats were fasted 36 hours and adrenalectomized rats 12 hours prior to phlorrhizination. An olive oil suspension of 5% pulverized phlorrhizin was injected subcutaneously. An initial dose of 2 cc. of the solution was followed by subsequent doses of 1.5 cc. at 12-hour intervals. The adrenalectomized rats were given two-thirds of this dose of phlorrhizin. The adrenalectomized rats, treated with extract, however, were given the same dose of phlorrhizin as the normal rats. During the first 6 hours of each 12-hour period the rats were injected intraperitoneally three times with a dose of 11 cc. each, of the solution to be tested. The solutions used in the experiments on glycogenesis contained approximately equivalent quantities of sodium and potential carbohydrate as follows.

Glucose =0.67% NaCl +1.5% glucose
Lactate =1.5% *d* lactic acid neutralized with NaOH
Pyruvate =1.5% pyruvic acid neutralized with NaOH
Alanine =0.45% NaCl +1.5% *d* or *l* alanine

In some animals, both normal and adrenalectomized, abdominal distension persisted following the intraperitoneal injections of these solutions. These animals were excluded in reporting the results of the experiments.

The specimens of rat urine were preserved by the addition of thymol and sodium fluoroide Determinations of lactic acid (method of Friedemann & Kendall, 5), however, were made only on urine specimens preserved by freezing. Glucose analyses on urine specimens were made with Benedict's quantitative reagent. Non-protein nitrogen was determined by the Kjeldahl method after protein had been precipitated with sodium tungstate and sulfuric acid. Urine amino acids were determined by the colorimetric method of Folin (6). Qualitative estimation of ketone bodies in urine specimens was made with sodium nitroprusside.

OBSERVATIONS

Phlorrhizin Treatment

Hypoglycemia. Normal dogs and rats displayed no signs of hypoglycemia during phlorrhizin treatment. In contrast to normal animals, adrenalectomized, phlorrhizin treated animals showed marked signs of hypoglycemia. In dogs, ocular palsy, diarrhea, vomiting, convulsive movements and semiconsciousness were observed. In rats prostration was followed by severe convulsion.

Treatment with 2 cc. of aqueous adrenal cortical extract, injected intraperitoneally at 6-hour intervals (a total of 8 cc. per day) completely prevented signs of hypoglycemia in adrenalectomized, phlorrhizin treated rats. The subcutaneous administration of 40 cc. of adrenal cortical extract ameliorated the signs of hypoglycemia in a fasted, adrenalectomized, phlorrhizin treated dog. Treatment with 0.5 mg. of 17-hydroxy-11-dehydrocorticosterone in oil injected subcutaneously at 12-hour intervals (a total of 1.0 mg. per day) was effective in preventing convulsions in adrenalectomized, phlorrhizin treated rats if treatment was begun 12 hours prior to the first injection of phlorrhizin. Treatment with 2.0 mg. of desoxycorticosterone acetate in oil injected subcutaneously at 12-hour intervals (a total of 4.0 mg. per day) did not prevent the appearance of signs of hypoglycemia in adrenalectomized, phlorrhizin treated rats.

[7] The adrenal cortical extract was supplied through the courtesy of Doctor David Klein, Wilson Laboratories, Chicago, Ill.

[8] The crystalline corticosterone and 17-hydroxy-11-dehydrocorticosterone (compound *E*) were generously provided by Doctor E. C. Kendall of the Mayo Clinic, Rochester, Minn.

Lowered threshold for hypoglycemic signs. The fact that adrenalectomized, phlorrhizin treated dogs displayed signs of hypoglycemia at a blood glucose[9] level at which no signs of hypoglycemia were observed in normal, phlorrhizin treated dogs, indicated a lowered threshold in the adrenalectomized animals (table 1).

TABLE 1. EFFECT OF ADRENALECTOMY ON THE THRESHOLD FOR HYPOGLYCEMIC SIGNS IN PHLORRHIZIN TREATED DOGS

Animal used	Hours of phlorrhizin treatment	Blood glucose
		mg./100 cc.
Adrenalectomized, phlorrhizin treated	7	71
	10	62[1]
	12	66
	15	54[1]
	21	56
Normal control, phlorrhizin treated	30	52
	31	59
	36	54
	41	56

[1] Denotes the occurrence of hypoglycemic convulsions.

Gluconeogenesis. Adrenalectomized, phlorrhizin treated rats excreted less glucose and nitrogen than phlorrhizin treated controls (fig. 1). Furthermore the rate of glucose and nitrogen excretion decreased much more rapidly in the adrenalectomized, phlorrhizin treated rats than in phlorrhizin treated controls. Injections of glucose ameliorated the hypoglycemia and prevented the rapid fall in nitrogen excretion of adrenalectomized, phlorrhizin treated rats (table 2). It seems probable that the rapid rate at which nitrogen excretion diminished in adrenalectomized, phlorrhizin treated rats was directly related to the rapidity with which hypoglycemia developed in these animals.

TABLE 2. EFFECT OF GLUCOSE TREATMENT ON NITROGEN EXCRETION OF FASTED, ADRENALECTOMIZED, PHLORRHIZIN TREATED RATS

Animal used	Treatment	Mg. nitrogen/100 gm. body wt./24 hours		
		12–24th hr.	24–36th hr.	36–48th hr.
Adrenalectomized, phlorrhizin treated	Normal saline	84 (3)	42 (3)	22 (3[1])
Adrenalectomized, phlorrhizin treated	Glucose	—	74 (7)	100 (2)
Normal controls, phlorrhizin treated	Normal saline	145 (3)	123 (3)	83 (3)

The numbers in parentheses indicate the number of rats used in each experiment.
[1] Two of these rats died in hypoglycemic convulsion.

Glucose:nitrogen ratio. The glucose:nitrogen ratio was consistently and significantly lower in adrenalectomized, phlorrhizin treated rats, than in phlorrhizin treated controls (fig. 1). These observations confirm the studies of Evans (7). Treatment with alanine altered little the glucose:nitrogen ratio (table 3) although it increased the excretion of nitrogen and glucose. This suggests that the abnormally low glucose:nitrogen ratio observed in adrenalectomized, phlorrhizin treated rats was the result primarily of a deficiency of adrenal cortical hormone, rather than the result of hypoglycemia.

[9] For convenience all blood sugar values determined by the modified micro method of Folin and Malmros are arbitrarily referred to in this study as 'blood glucose values.'

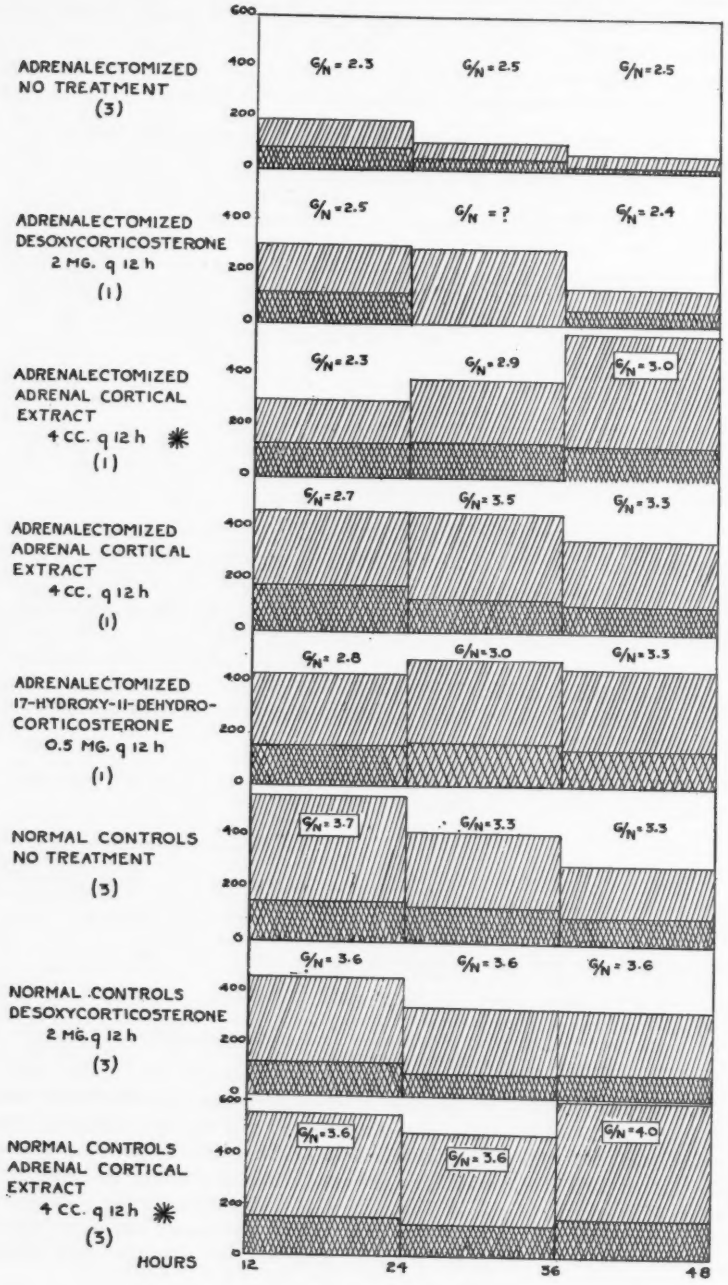

Fig. 1. (*See next page for legend.*)

TABLE 3. EFFECT OF ALANINE TREATMENT ON THE GLUCOSE:NITROGEN RATIO OF ADRENALECTOMIZED, PHLORRHIZIN TREATED RATS

Animal used	Treatment	Glucose excretion	Nitrogen excretion	Glucose:Nitrogen ratio
		mg./100 gm./24 hr.	mg./100 gm./24 hr.	
Adrenalectomized and phlorrhizin treated (3)	None	148	64	2.3
Adrenalectomized and phlorrhizin treated (4)	Alanine 1.0 gm.	420	160[1]	2.6
Normal controls, phlorrhizin treated (3)	None	488	132	3.7
Normal controls, phlorrhizin treated (3)	Alanine 1.0 gm.	670	204[1]	3.3

[1] Nitrogen excretion refers to the total non-protein nitrogen excretion from which the amino acid nitrogen excretion has been deducted.

Glucose and nitrogen excretion of adrenalectomized, phlorrhizin treated rats was restored to normal by treatment with adrenal cortical extract or 17-hydroxy-11-dehydrocorticosterone (fig. 1). The glucose and nitrogen excretion of normal, phlorrhizin treated rats was also increased by treatment with adrenal cortical extract. In both normal, phlorrhizin treated and adrenalectomized, phlorrhizin treated rats adrenal cortical hormone therapy was followed by a significant rise in the glucose:nitrogen ratio. Treatment with desoxycorticosterone acetate did not prevent the hypoglycemic convulsions and did not restore the glycosuria or glucose:nitrogen ratio of adrenalectomized, phlorrhizin treated rats to normal.

Glucose utilization. Since little or no glucose is stored in phlorrhizin treated animals it is possible to calculate the per cent of injected glucose that is utilized by applying formula 1.

$$\begin{pmatrix}\%\text{ of injected}\\ \text{glucose that}\\ \text{is utilized}\end{pmatrix} = (100) - (100)\left(\frac{\begin{pmatrix}\text{Total glucose}\\ \text{excreted}\end{pmatrix} - \begin{pmatrix}\text{Total nitro-}\\ \text{gen excreted}\end{pmatrix}\begin{pmatrix}\text{Fasting}\\ \text{G/N ratio}\end{pmatrix}}{(\text{Glucose injected})}\right)$$

Applying this equation it was observed that a high percentage of glucose was utilized in the adrenalectomized, phlorrhizin treated rats (table 4). Adrenalectomized, phlorrhizin treated rats utilized 43% of the injected glucose as compared with 19% utilized by normal, phlorrhizin treated rats. If the actual glucose:nitrogen ratio of 2.5 is used instead of the normal ratio of 3.3, the utilization of glucose amounted to 31%.

Since, in experiments where no glucose was injected, the glucose:nitrogen ratio of fasting, adrenalectomized, phlorrhizin treated rats was 25% lower than the glucose:nitrogen ratio of fasting, normal, phlorrhizin treated rats, it appeared unlikely that the increased utilization of glucose in this experiment could be explained by differences in the absorption of glucose from the peritoneal cavity.

Treatment with adrenal cortical extract prevented the increase in glucose utilization of adrenalectomized, phlorrhizin treated rats (table 4). Treatment with desoxycorticosterone acetate was not effective in this respect.

FIG. 1. THE EFFECT OF ADRENALECTOMY AND TREATMENT WITH ADRENAL CORTICAL HORMONE ON GLUCOSE AND NITROGEN EXCRETION IN PHLORRHIZIN TREATED RATS. Mg. glucose/100 gm. body wt./24 hr., total shaded area; mg. nitrogen/100 gm. body wt./24 hr., darker shaded area. *Hormone treatment started 12 hours after instead of before phlorrhizin treatment.

TABLE 4. EFFECT OF ADRENALECTOMY AND ADRENAL CORTICAL HORMONE TREATMENT ON THE UTILIZATION OF INJECTED GLUCOSE IN PHLORRHIZIN TREATED RATS

Animal used	Hormone treatment, 12 hr.	Glucose excretion	Nitrogen excretion	Glucose utilized (G/N=2.5)	Glucose utilized (G/N=3.3)
		mg./12 hr.	mg./12 hr.	%	%
Adrenalectomized, phlorrhizin treated (9)	None	532	75	31	43
Adrenalectomized, phlorrhizin treated (3)	Desoxycorticosterone 2 mg.	585	103	35	51
Adrenalectomized, phlorrhizin treated (1)	Adrenal cortical extract 4 cc.	860	115	—	4
Normal controls, phlorrhizin treated (7)	None	812	127	—	19

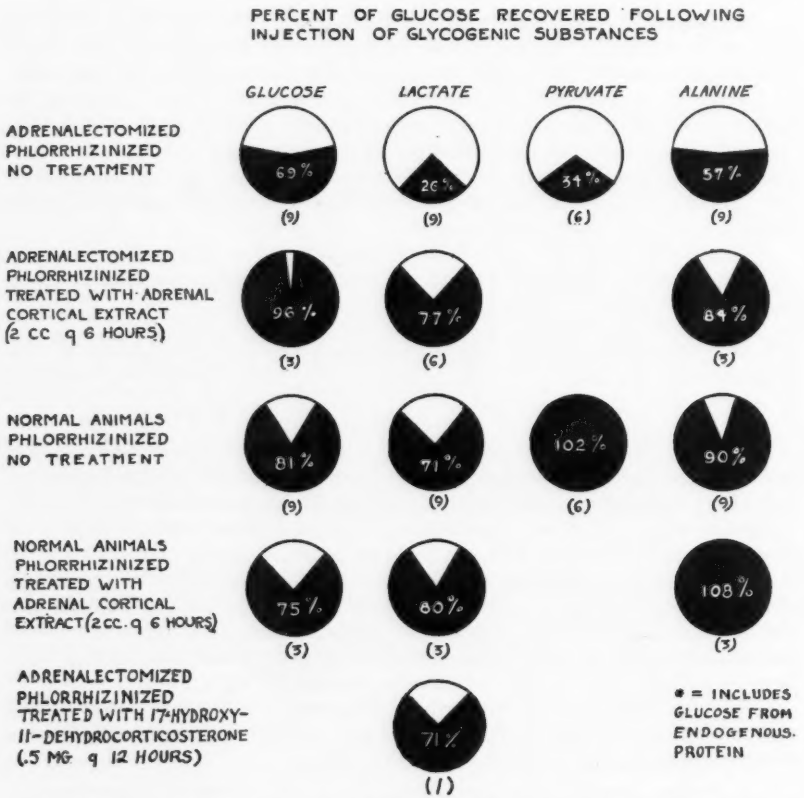

Fig. 2. EFFECT OF ADRENALECTOMY AND TREATMENT WITH ADRENAL CORTICAL HORMONE ON PERCENTAGE RECOVERY OF GLUCOSE FOLLOWING THE INJECTION OF GLYCOGENIC SUBSTANCES IN PHLORRHIZIN-TREATED RATS.

Glycogenesis. Data on the excretion of glucose derived from endogenous protein, injected alanine, and injected glucose have been presented, (table 2 and 3). The glycogenic action of lactic and pyruvic acids has also been studied (fig. 2). Lactic acid, glucose and pyruvic acid solutions were injected respectively in successive 12-hour, periods during one experiment and in the reverse order during a second experiment. With each group of untreated adrenalectomized rats a series of normal, phlorrhizin treated controls were injected with the same glycogenic substances. The per cent of potential glucose that was recovered following the injection of lactic acid was calculated by formula 2.

$$\begin{pmatrix}\%\text{ of potential}\\ \text{glucose injected}\\ \text{that was recovered}\end{pmatrix} = (100)\left(\frac{\begin{pmatrix}\text{Total glucose}\\ \text{excreted}\end{pmatrix} - \begin{pmatrix}\text{Total nitro-}\\ \text{gen excreted}\end{pmatrix}\begin{pmatrix}\text{Fasting}\\ \text{G/N ratio)}\end{pmatrix}}{\text{(Potential glucose injected)}}\right)$$

In the adrenalectomized, phlorrhizin treated rats the proportion of potential glucose recovered amounted to 26% as compared to 71% in the normal, phlorrhizin treated rats (fig. 2). The recovery following the injection of pyruvic acid was 34% in the adrenalectomized rats as compared to 102% in the normals.

The apparent decrease in glucose formation from lactic acid which was observed in adrenalectomized, phlorrhizin treated rats was not due to excessive excretion of lactic acid since less than 5% of the total quantity of injected lactic acid was found in the urine. Following the injection of 610 mg. of lactic acid, the average urinary excretion of lactic acid was 29 mg. in 3 adrenalectomized, phlorrhizin treated rats and 45 mg. in 3 normal, phlorrhizin treated rats.

Treatment with adrenal cortical extract or 17-hydroxy-11-dehydrocorticosterone restored the ability of adrenalectomized, phlorrhizin treated rats to form glucose from lactic acid (fig. 2). Desoxycorticosterone acetate treatment was ineffective in this respect.

Ketonuria. It is interesting to note that the ketonuria of fasting, adrenalectomized, phlorrhizin treated rats was increased by the administration of adrenal cortical extract or 17-hydroxy-11-dehydrocorticosterone, (table 5). The ketonuria of a fasting,

TABLE 5. EFFECT OF ADRENALECTOMY AND ADRENAL CORTICAL HORMONE TREATMENT ON THE KETONURIA OF FASTED PHORRHIZIN TREATED RATS

Animal used	Hormone treatment, 12 hr.	Qualitative estimation of ketonuria		
		12–24th hours	24–36th hours	36–48th hours
Adrenalectomized, phlorrhizin treated (3)	None	o	o	o
Adrenalectomized, phlorrhizin treated (1)	Desoxycorticosterone 2 mg.	++	++	o
Adrenalectomized, phlorrhizin treated (2)	17-hydroxy-11-dehydrocorticosterone 0.5 mg.	o	++	++
Adrenzlectomized, phlorrhizin treated (2)	Adrenal cortical extract 4 cc.	++++	+++	++
Normal controls, phlorrhizin treated (4)	None	++	+++	+++
Normal controls, phlorrhizin treated (2)	Adrenal cortical extract 4 cc.	++	+++	++++

adrenalectomized, phlorrhizin-treated dog was also increased by adrenal cortical extract treatment. Desoxycorticosterone acetate treatment had less effect upon ketonuria than either adrenal cortical extract or 17-hydroxy-11-dehydrocorticosterone. It is impossible to interpret the significance of ketonuria in the absence of data on ketonemia. However, the increase in glucose and nitrogen excretion with an increase in glucose:nitrogen ratio suggests that the increased ketonuria associated with adrenal cortical hormone treatment was due to an effect on carbohydrate utilization as well as on protein conversion.

Insulin Treatment

Hypoglycemia. The intravenous injection of 0.25 U of insulin per kg. was followed by severe convulsions in adrenalectomized dogs. No signs of hypoglycemia were observed in normal dogs following similar treatment. There was also a greater reduction of blood glucose in the adrenalectomized, insulin-treated dogs (fig. 3).

All signs of hypoglycemia in the adrenalectomized, insulin treated dog were prevented by the administration of 70 cc. of adrenal cortical extract injected subcutaneously and intravenously during a 12-hour period preceding the injection of insulin. Convulsions were prevented, although a mild hypoglycemia reaction occurred, by treatment with 12 mg. of 17-hydroxy-11-dehydrocorticosterone dissolved in oil and injected subcutaneously during a 12-hour period preceding the injection of insulin. Treatment with desoxycorticosterone (maintenance dose for life supplemented with 20 mg. injected intramuscularly) had no demonstrable effect upon the blood glucose level or upon the hypoglycemic convulsions, following insulin treatment, in adrenalectomized dogs.

Lowered threshold for hypoglycemic signs. Following insulin treatment it was observed that normal dogs and extract treated adrenalectomized dogs did not show hypoglycemic convulsions at a blood glucose level which was associated with marked signs of hypoglycemia in untreated or desoxycorticosterone acetate treated adrenalectomized dogs (fig. 3).

Glycogen. The subcutaneous injection of 0.25 mg. per kg. of epinephrin was followed by a decreased glycemic response in adrenalectomized dogs maintained on desoxycorticosterone acetate, (fig. 3). However, a comparison of the glycemic response revealed the fact that the adrenalectomized, desoxycorticosterone treated dogs had a higher than normal blood glucose level during the first hour of the test and a lower than normal blood glucose level during the third and fourth hours of the test. It appeared from these studies that the decreased glycemic response of these animals was not due to an insensitivity to epinephrin, because of the immediate rise in blood glucose to a level higher than normal following epinephrin treatment. The less sustained glycemic response of these animals however, could be accounted for by a decrease in the quantity of available glycogen or an increase in the utilization of available glucose.

Following the combined epinephrin and insulin test there was a decreased glycemic response in the adrenalectomized, desoxycorticosterone acetate treated dogs (fig. 3). The results of the combined test corroborate the changes observed in the insulin test and in the epinephrin test.

Glycogenesis. Although it would have been desirable to have been able to have used phlorrhizin treated, adrenalectomized dogs in a study of glycogenesis following the administration of lactic, pyruvic and amino acids, it was found to be impossible because of the occurrence of marked hypoglycemic reactions associated with vomiting, diarrhea and collapse. For this reason a combined insulin and carbohydrate test was devised in which insulin was given 15 minutes after the start of the intravenous in-

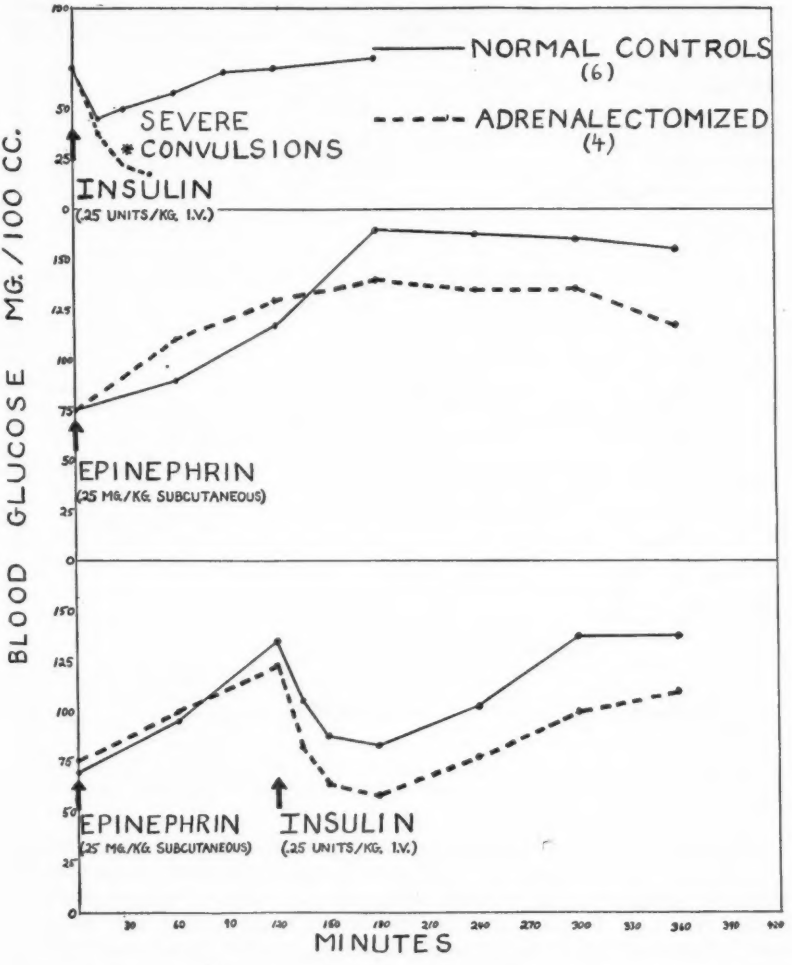

Fig. 3. Effect of epinephrin and insulin on blood glucose levels of normal dogs and adrenalectomized dogs maintained with pellets of desoxycorticosterone acetate.

fusion of the glycogenic substance. When 4.5 gm. of glucose were injected all signs of hypoglycemia in the insulin treated adrenalectomized dog were prevented (fig. 4). However, the injection of 9 gm. of racemic lactic acid did not prevent the occurrence of severe hypoglycemic convulsions following the standard dose of insulin. It has been shown that the administration of lactic acid did not relieve the signs and symptoms of hypoglycemia in a patient with Addison's disease (2).

Blood glucose. No constant difference was noted between the fasting blood glucose level of adrenalectomized, desoxycorticosterone acetate treated and normal dogs. However, treatment with a large dose of adrenal cortical extract was followed by a rise in the fasting blood glucose level (table 6).

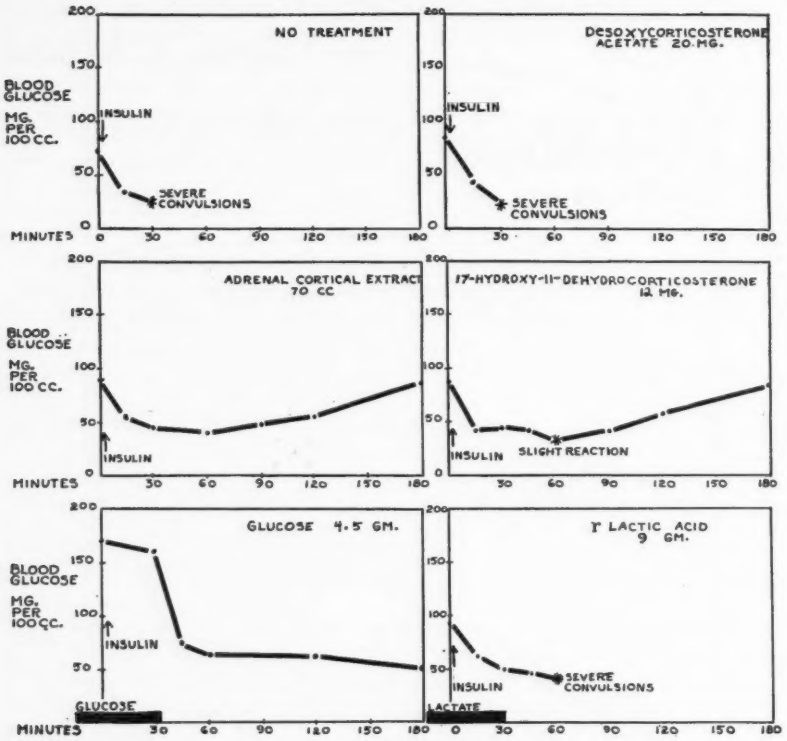

Fig. 4. Effect of injected glucose and lactic acid and effect of adrenal cortical hormone therapy on blood glucose of adrenalectomized dog (211) following intravenous administration of insulin (0.25 u/kg.).

Respiratory quotient. A slightly increased respiratory quotient has been reported in untreated, fasting, adrenalectomized rats (7). It has also been shown that the respiratory quotient of fed, adrenalectomized rats was lowered by treatment with cortical extract (1). Furthermore it has been observed that patients with Addison's disease frequently had a higher fasting respiratory quotient than normal subjects maintained on a similar diet (2). The respiratory quotient of these patients was re-

Table 6. Effect of adrenal cortical hormone treatment on the blood glucose, urinary excretion of nitrogen, and respiratory quotient of fasted adrenalectomized dog 211

Hormone treatment and dose	Blood glucose	Nitrogen excretion	Respiratory quotient[1]
	mg./100 cc.[1]	gm./hour[1]	
No treatment	87	2.50	76
Desoxycorticosterone, 50 mg.	86	3.28	76
Corticosterone, 15 mg.	89	3.50	74
Adrenal cortical extract, 50 cc.	96	3.90	72

[1] These values represent the average of 4 determinations obtained during a 6-hour period of study.

duced by treatment with adrenal cortical extract, 17-hydroxy-11-dehydrocorticosterone or corticosterone (2). The observations in the present study show a similar but less marked elevation of the respiratory quotient of fasted, adrenalectomized desoxycorticosterone acetate treated dogs and a similar but less marked fall after treatment with adrenal cortical extract or corticosterone (table 6).

Nitrogen excretion. Treatment with adrenal cortical hormone was followed by an increase in the nitrogen excretion of fasting, adrenalectomized dogs. Adrenal cortical extract treatment appeared to have the most marked effect in this respect (table 6).

DISCUSSION

In 1936 Buell, Anderson and Strauss showed that adrenalectomized animals deposited very little glycogen in the liver following the ingestion of lactic acid, unless treated with adrenal cortical extract (8). Similar studies following glucose ingestion were not performed at this time, hence it was not known whether this defect represented primarily an inability of adrenalectomized animals to form glucose from lactic acid, or whether the defect was merely an inability to deposit glucose as glycogen. In 1937 Deuel, Hallman, Murray and Samuels showed that adrenalectomized rats were able to convert glucose to liver glycogen at the same rate as normal rats (9) Our experiments indicated that adrenalectomized rats, maintained in good condition with sodium chloride or desoxycorticosterone acetate, were limited in their ability to form glucose from lactic, pyruvic and glycogenic amino acids. The administration of adrenal cortical extract or 17-hydroxy-11-dehydrocorticosterone restored the ability to form glucose from lactic, pyruvic and glycogenic amino acids.

Following glucose injections it was observed that the glucose utilization of adrenalectomized, phlorrhizin treated rats was greater than that of phlorrhizin treated controls. Furthermore, treatment with adrenal cortical hormone decreased the utilization of glucose in adrenalectomized, phlorrhizin treated rats. Indirect evidence of the increased utilization of glucose in adrenal cortical insufficiency was indicated by (*a*) the decreased glucose:nitrogen ratio in fasting adrenalectomized, phlorrhizin treated rats and (*b*) the high standard respiratory quotient of patients with Addison's disease. Adrenal cortical hormone therapy (*a*) increased the glucose:nitrogen ratio and the ketonuria of fasted adrenalectomized, phlorrhizin treated rats and (*b*) raised the blood glucose and lowered the standard respiratory quotient of adrenalectomized dogs and patients with Addison's disease (2).

It has long been known that hypoglycemia occurs with great frequency in adrenalectomized animals. The ease with which hypoglycemic signs and symptoms can be induced by prolonged fasting (2), by treatment with insulin or phlorrhizin, by feeding a diet adequate in calories but low in carbohydrate (2) and by the administration of a standard glucose tolerance test (2) has been demonstrated. Until recently little attention has been given to the fact that the threshold for the occurrence of hypoglycemic signs and symptoms is lowered in adrenalectomized animals and in patients with Addison's disease (2). It has been shown that the administration of adrenal cortical hormone raises the threshold for hypoglycemic signs.

The low glucose:nitrogen ratio of adrenalectomized, phlorrhizin treated rats was described by Evans in 1936 (7), although not confirmed by Wells (10). In the present study the low glucose:nitrogen ratio was one of the most constant and striking findings in adrenalectomized, phlorrhizin treated rats. The administration of adrenal cortical hormone not only raised the glucose:nitrogen ratio of adrenalectomized, phlorrhizin treated rats but also increased the ratio in normal, phlorrhizin treated animals.

SUMMARY

Abnormalities in carbohydrate metabolism observed in adrenalectomized, phlorrhizin treated rats maintained by means of sodium chloride or desoxycorticosterone acetate therapy were: (*a*) an impaired ability to form glucose from lactic acid, pyruvic acid, or alanine, (*b*) an increased utilization of available glucose, (*c*) a decreased ketonuria, glycosuria, nitrogen excretion, and glucose:nitrogen ratio.

Adrenalectomized dogs maintained in electrolyte balance by treatment with desoxycorticosterone acetate were observed to have: (*a*) a decreased glycemic response to epinephrin and an increased sensitivity to insulin, (*b*) a decreased threshold for signs of hypoglycemia induced by insulin or phlorrhizin treatment.

The administration of 17-hydroxy-11-dehydrocorticosterone or adrenal cortical extract to adrenalectomized, phlorrhizin treated rats resulted in: (*a*) a restoration of the ability to form glucose from lactic acid, pyruvic acid and alanine, (*b*) a decreased utilization of available glucose, (*c*) an increased ketonuria, glycosuria, nitrogen excretion and glucose:nitrogen ratio.

The administration of corticosterone or adrenal cortical extract to adrenalectomized dogs resulted in a lowered respiratory quotient, an increased nitrogen excretion, and an increased blood glucose level.

The administration of 17-hydroxy-11-dehydrocorticosterone or adrenal cortical extract to adrenalectomized dogs resulted in an increase in the threshold for signs of hypoglycemia induced by treatment with insulin or phlorrhizin.

CONCLUSION

The ability of adrenalectomized animals to convert three carbon atom intermediate substances of carbohydrate and protein metabolism to glucose or glycogen is markedly impaired. This defect may account for the rapid development of, and low threshold for, hypoglycemia which follows a prolonged fast, insulin or phlorrhzin treatment. Impairment in the ability to resynthesize glycose or glycogen is associated with an increase in the utilization of these substances. The increased utilization of available glucose is reflected in the decreased glucose:nitrogen ratio of adrenalectomized, phlorrhizin treated animals. The increased utilization of carbohydrate and the decreased ability to form new glucose from the intermediate products of carbohydrate and protein metabolism leads to the rapid exhaustion of glycogen stores in fasting adrenalectomized animals. The administration of adrenal cortical extract of 17-hydroxy-11-dehydrocorticosterone restores the ability of adrenalectomized rats and dogs to resynthesize glucose and glycogen from intermediate products of carbohydrate and protein metabolism and corrects the defects in carbohydrate metabolism.

The authors are indebted to Harry Eisenberg and William Sause for their technical assistance in this study.

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MODIFICATION OF THE INTRAUTERINE ASSAY METHOD FOR PROGESTERONE[1]

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THE SEVERAL KNOWN ACTIONS of progesterone[2] on the uterus have naturally been applied as criteria for testing the presence of the hormone in the animal body. Several unique methods of bioassay have been proposed, each utilizing one or more of the varied phenomena of uterine progestational activity as its physiological basis. The action of progesterone on uterine motility has been suggested by Van Dyke and Chen (1) as a method of progesterone assay. The optimum amount to be tested is about 0.45 mg. of progesterone. Astwood (2) suggests a test for 0.50 to 1.0 mg. of progesterone by observing the decidual reaction in rats. Pincus and Werthessen (3) utilize as a criterion of a bioassay method the diameter of an ovum and ratio between the area of the epithelium and stroma of the rabbit's uterus. Duyvene de Wit (4), by measuring the length of the ovipositor of the bitterling can detect the presence of minute amounts of the sex hormones, and states that by exact observation of the rate of growth of the ovipositor he can distinguish between the various hormones and thus assay any one of them, for example progesterone.

The most widely used criterion has been that of histological alteration in the uterus, described as progestational proliferation. Corner and Allen (5) observed histological changes in the uterus of the mature oöphorectomized rabbit upon daily subcutaneous injection over a period of 5 days of equal parts of a solution containing progestin. They suggested a scale of reactions from + to + + + + with which to describe the progestational proliferation of the observed uterus. A + + + reaction has been found to represent the activity of approximately 1 mg. of crystalline progesterone. Clauberg (6) and later McPhail (7) modified the Corner-Allen technic by using immature female rabbits primed with estrogen. After several daily intramuscular injections of progestin the uterus is examined histologically and graded on the basis of the + to + + + + system. By the expedient of using immature rabbits it was found that the sensitivity to progesterone was doubled, i.e., 0.5 mg. of progesterone produced a + + + reaction as compared to 1 mg. of the hormone necessary with the Corner-Allen technic.

Fournier, Albrieux and Grosso (8) describe experiments in which progesterone was injected into the uteri of estrogen primed immature rabbits. Single doses of 0.5 mg. of progesterone applied to the uterus produced a + + + proliferation as compared to the + to + + reaction brought about by 0.5 mg. of progesterone in a single subcutaneous injection. McGinty, Anderson and McCullough (9) observed that intrauterine application of amounts of progesterone as small as 0.13 γ to the estrogen primed immature rabbit caused a recognizable progestational proliferation. The reaction was not proportional to the several doses

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[2] The progesterone in this experiment was generously supplied through the courtesy of Dr. Erwin Schwenk of the Schering Corporation.

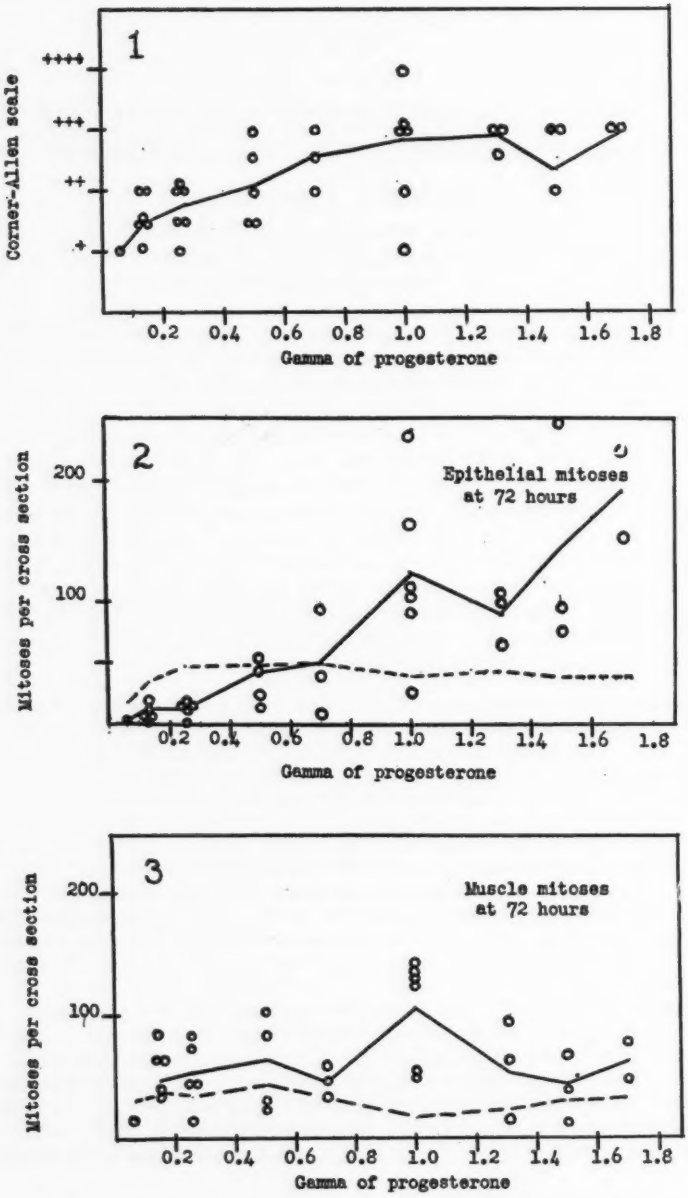

Fig. 1–3. EXPERIMENTAL SEGMENT, SOLID LINE; CONTROL SEGMENT, BROKEN LINE.

of progesterone used and hence was not applicable as a quantitative test. Because of the sensitivity obtained with the intrauterine bioassay method, the following experiments were designed in an attempt to place the method on a quantitative basis.

The method as described by McGinty, Anderson and McCullough consists of priming immature female rabbits with 25 I.U. of estrogen per day for 6 days. On the seventh day the animal's abdomen is opened and 0.1 cc. of a solution of peanut oil and progesterone is applied to the lumen of a uterine segment 2 to 3 cm. in length, isolated by double ligatures. The animal is killed 72 hours after this operation. The uterine segment is examined histologically and the progestational proliferation observed is described on the basis of the Corner-Allen scale.

Thirty-six animals were treated as suggested by McGinty, Anderson and McCullough. A 0.1 cc. solution of peanut oil, containing from 0.06 to 1.7γ of crystalline progesterone, was applied to one uterine segment 2 cm. in length in each animal. Control segments were taken in the opposite uterine horn and to each was applied 0.1 cc. of peanut oil. The progestational proliferation induced by the several doses of crystalline progesterone is indicated in figure 1. The control horn in no case exhibited a recognizable proliferation. The results indicate that the mean value for each dosage level increases as the dose increases, but that each dose produces a variety of reactions.

Following the suggestion of Pincus and Werthessen (3) the area of glandular mucosa and the area of stroma were measured with the planimeter. The proportion between the two was derived mathematically. These measurements on several uteri failed to show significant results when compared to the dose of progesterone applied.

Since it has been shown (10), that one of the specific effects of progesterone is to induce mitotic division in the uterine epithelium, and also in the myometrium (11), mitotic counts of both epithelium and muscle were made on cross sections, 10 microns thick. The counts, which are indicated in figures 2 and 3, show that the rate of mitotic division in the epithelium is the most constant criterion of progestational action.

To further discover the action of locally applied progesterone, the effect of several dosage levels, 0.13, 0.25, 0.50, and 1.0γ on a group of 113 animals was explored from 24 hours to 96 hours after application. Cross sections, 10 microns thick were graded on the basis of the Corner-Allen scale and were also counted for mitoses in the epithelium and in the myometrium. The mitotic counts are indicated in figures 4 to 11, and were made on one cross section, for cross sections from different regions in the same uterine segment showed very little variation in either muscle or epithelial mitotic counts. All progesterone injected segments exhibited positive progestational proliferation after 48 hours. Control segments were taken from the opposite uterine horn and were untreated.

RESULTS AND DISCUSSION

The effect of locally applied progesterone as interpreted on the basis of the Corner-Allen scale, in terms of progestational proliferation, is not shown graphically. There was, however, with each of the several doses of progesterone observed, a gradual rise from no proliferation to a maximum and then a diminution or retrogression of observed reactions over a period of 96 hours. With the smaller doses the peak of progestational proliferation occurred before 72 hours and with the larger doses the peak occurred at or after 72 hours.

This effect may be at least a partial explanation of the inaccuracy of this method, as described by McGinty et al., as a bioassay technic. For at 72 hours, at which time the animal is killed, the effect of small doses of progesterone would be retrogressing and the effect of large doses would not as yet have reached a maximum. It would be

possible for a large dose to cause the same degree of progestational proliferation as a small dose because the effect of each was observed simultaneously, rather than at the optimum reaction time for the given dose.

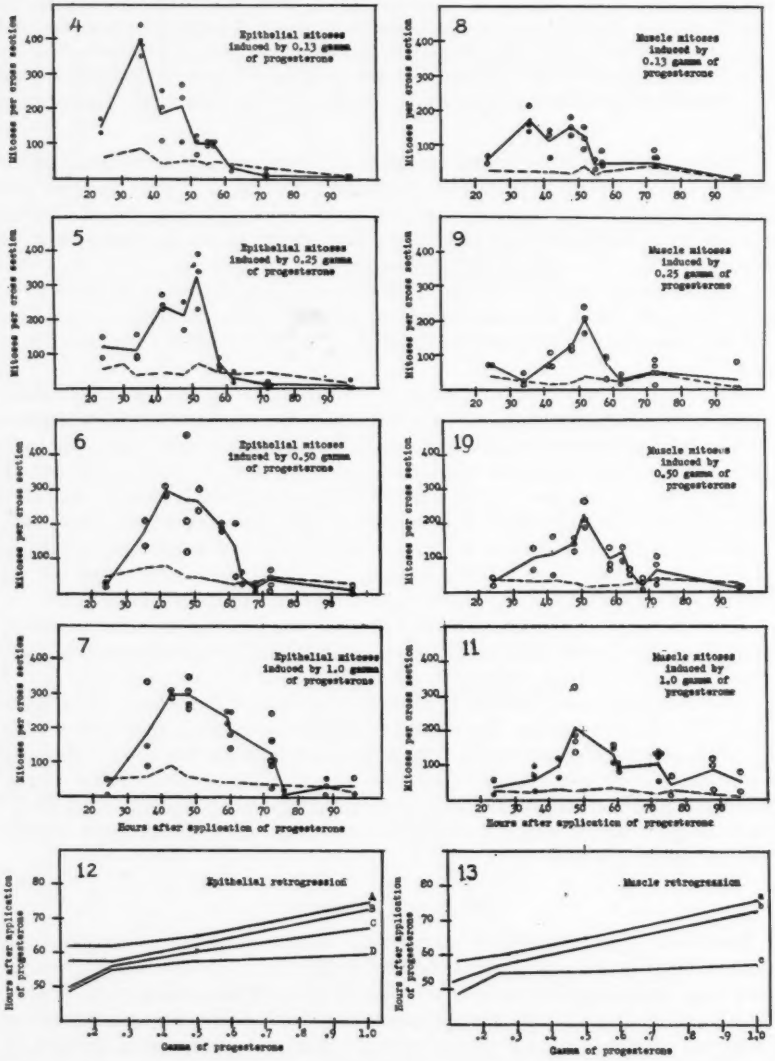

Fig. 4–13. (A, RETROGRESSION OF EPITHELIAL MITOSES TO 50 PER CROSS SECTION, B to 100, C to 150, D to 200; a, RETROGRESSION OF MUSCLE MITOSES TO 50 PER CROSS SECTION, b to 100, c to 150.)

The curves obtained from plotting the epithelial mitotic counts against time after the application of progesterone are all roughly parabolic. It was found, however, that the height of the individual curves was extremely variable, but the major effect of

increasing doses was to increase the time of the progestational action as interpreted by mitoses. The essential consistent difference found to vary with the amount of applied progesterone, is the time necessary for the mitoses in the progesterone injected segment to fall below the level of the control epithelial and myometrial mitotic counts. Further analysis of the individual curves of epithelial mitotic division indicates that with the smaller doses of progesterone the mitoses retrogress to given levels, such as 200, 150, 100 and 50 mitoses per cross section, sooner than with larger doses. This is illustrated in figure 12.

The effect of progesterone on myometrial mitoses follows the same basic pattern as its effect on epithelial mitoses. There is, however, a general rise in muscle mitoses of both the control and the progesterone injected segments at 72 hours. Since the 72-hour controls are the only ones treated with peanut oil and hence the only control segments which were subjected to distention, this may be an indication of the muscle hyperplasia which accompanies uterine distention. It may be possible that the distention factor plays a further role in inducing muscle mitoses not recognizable in these data. For this reason it seems probable that the most constant method of evaluating progestational activity would be that of epithelial mitotic counts. A chart of the retrogression of muscle mitosis, figure 13, with points at 150, 100, 50 mitoses per

TABLE 1. SAMPLE ASSAY OF UNKNOWN SOLUTIONS

Sample No.	Hours after application	Reaction, Corner-Allen scale	Epithelial mitoses	Muscle mitoses	Estimation of progesterone from epithelial mitoses	Estimation of progesterone from muscle mitoses	Final estimate γ	Actual amount, γ
1	60	++	234	112	more than 1γ	more than 0.38γ		
	72	++	23	19	less than 0.86γ	less than 0.85γ	0.38–0.86	1.00
2	60	++–+++	134	52	0.38–0.46γ	0.25–0.38γ		
	72	++	70	20	0.86–0.95γ	less than 0.85γ	0.25–0.46	0.25
3	60	++	209	115	more than 1γ	more than 0.38γ		
	72	++–+++	43	46	less than 0.86γ	less than 0.85γ	0.38–0.86	0.50

cross section, indicates that smaller doses of progesterone allow retrogression sooner than do the larger doses. In this respect the activity of muscle mitoses bears a resemblance to the activity of the epithelial mitoses.

The epithelial control counts with each dose of progesterone indicate a small rise in mitotic activity coincidentally with the observed peak of the progesterone injected segments. A similar rise was not observed in the counts of muscle mitosis. No control segment showed progestational proliferation that could be evaluated on the Corner-Allen scale.

To discover whether unknown solutions containing progesterone might be assayed using these data, three solutions were assayed, the contents of which were unknown until after the calculations were made and the result stated. The procedure was as follows.

Each of 2 animals primed with estrogen was applied with 0.1 cc. of the same peanut oil solution, by intrauterine injection. One animal was killed at 60 hours, the other at 72 hours. Counts of epithelial and muscle mitoses were made. The values were plotted in figures 12 and 13, and the amount of progesterone present was read directly from these graphs.

Table 1 indicates the results obtained from the assay of these 3 unknown solutions of peanut oil and progesterone. Although these results seemed encouraging at the time further work indicated complications arising from this method. Most important is the fact that doses higher than 2.0γ of progesterone no longer cause a response similar to that observed with doses less than 2.0γ. Mitotic counts of the uterine epi-

thelium and the myometrium of animals receiving more than 2.0γ of progesterone at 72 hours after application are not as high as might be expected; indeed, 9 of 10 cases have mitotic counts less than 50 in both the epithelium and myometrium.

This situation may well be likened to the reversal phenomena of progesterone at different dosage levels observed by Thomsen, Pedersen-Bjergaard and Anderson (12). It is apparent that the use of the modification described in this paper would be applicable as a quantitative bioassay method only when the progesterone content of a solution was below 17γ per cc. of peanut oil.

SUMMARY

The action of progesterone when applied to the uterus of the estrogen primed immature rabbit, on epithelial and muscle cell mitosis has been studied.

A quantitative bioassay of progesterone based on epithelial and muscle mitotic counts has been devised. The method is accurate only within arange of dosage for 0.06 to 1.7γ. Amounts of progesterone above 1.7γ are recognized qualitatively but not quantitatively.

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THE ATROPHY OF THE ADRENAL CORTEX FOLLOWING THE ADMINISTRATION OF LARGE AMOUNTS OF PROGESTERONE

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IT HAS BEEN SHOWN by several investigators that regressive morphological changes in certain endocrine glands can be induced by the administration of large amounts of the hormone normally secreted by the gland in question. Ingle and Kendall (1) and Ingle, Higgins and Kendall (2) have shown that the administration of large amounts of the hormone of the adrenal cortex and also, two crystalline compounds separated from the adrenal, which possess cortin-like activity, produced an atrophy of the adrenal cortex in the rat. Wells and Kendall (3) have shown that compounds separated from the adrenal cortex produced atrophy of the adrenal and thymus glands of rats when administered in large doses. These investigators have furthermore shown that a qualitative difference in the effect of these various compounds in atrophy of the adrenals does exist. It has been shown (4) that regressive changes occurred in the thyroid after the administration of large amounts of thyroid substance. It was also shown (5) that the injection of excessive amounts of follicular hormone or testicular hormone would produce regressive changes in the female or male gonads of rats.

The present report considers the results of a series of experiments designed to show the effect of the administration of large amounts of crystalline progesterone on the size and structure of the adrenal glands in male rats. A preliminary report of the results was published in abstract form (6).

METHODS

Male rats of the Yale strain, which weighed 175 to 180 gm., were used in the following series of experiments. They were kept in separate cages and maintained on a commercial diet (Purina Dog Chow). Occasional feedings of lettuce were also added. Before the experiments were started all the animals were observed for several days to determine whether or not a normal weight gain occurred on the above diet.

The animals were divided into experimental and control groups. The experimental animals were given 4.0 mg. of crystalline progesterone[1] in oil subcutaneously each day for 15 successive days. The control group was divided into 2 series. One series was injected with 4 cc. sesame oil daily for 15 successive days while the other control series was carried along as a normal control for a similar period.

Two days after the last injection all rats in the experimental and control group were weighed and killed by ether anesthesia or exsanguination. Both adrenals were carefully removed, weighed together to the nearest milligram and fixed and stained for histological study. The thymus gland was also removed, weighed and preserved

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[1] Thanks are due Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products Inc. for the generous supply of progesterone.

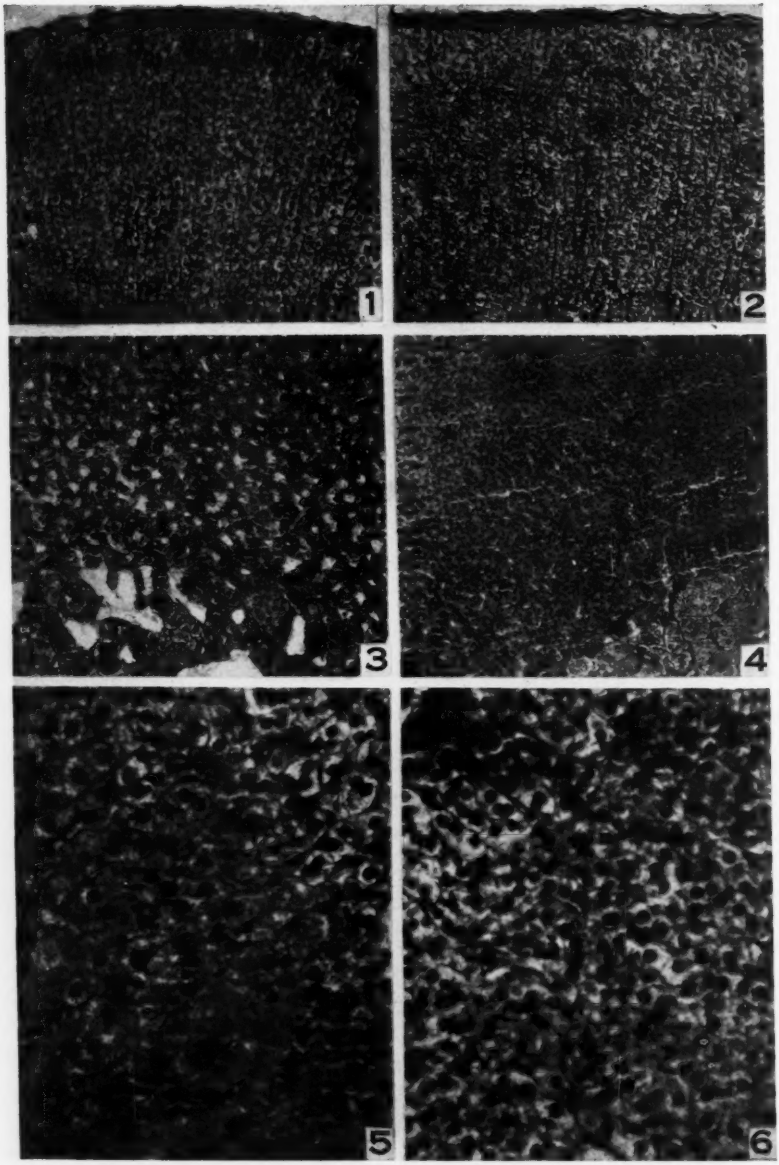

Fig. 1. A SECTION OF ADRENAL CORTEX SHOWING THE CAPSULE, ZONA GLOMERULOSA, AND ZONA FASICULATA OF A NORMAL CONTROL. ×100. Fig. 2. A SECTION OF THE ADRENAL CORTEX OF A PROGESTERONE INJECTED RAT AFTER 15 SUCCESSIVE INJECTIONS. Note difference in cell cytoplasm in the zona glomerulosa cells when compared to that of figure 1. ×100. Fig. 3. A SECTION THROUGH THE ADRENAL GLAND SHOWING THE ZONA RETICULATA AND A PART OF THE MEDULLA OF A NORMAL CONTROL. ×100. Fig. 4. A SECTION

(*Continued next page*)

for histological study. At this time the pituitary, pineal body, thyroid and the testes were removed for fixation and future microscopic studies. The removal of the adrenals and the separation of adherent connective and adipose tissue from them before weighing, evidently resulted in some loss of moisture and hence, necessarily involved an error in their weight values. Nevertheless, less error seemed to be involved by this method than by the removal of the capsule of each gland.

RESULTS

The data on body weight and changes in weight of the adrenal and thymus glands for each series of rats is shown in table 1. It can be noted that all 3 series, controls as well as experimentals, showed an average daily body weight gain of 3 to 4 gm. for the 17-day period. This gain was within the normal limits, and while the progesterone treated rats showed a slightly lower average, this difference was not within the border of statistical significance and hence must still be considered a normal variation.

TABLE 1. DATA RECORDED TO SHOW THE VARIOUS WEIGHT DIFFERENCES IN THE NORMAL AND PROGESTERONE INJECTED MALE RATS

Series	No. of animals	Body wt. gain	Adrenal wt.	Thymus wt.
		gm.	mg.	mg.
Normal controls	8	65.8	41.7	444
Sesame injected	10	66.0	39.1	360
Progesterone injected	10	57.4	30.9	293

As regards the differences in weight of adrenal glands, it can be seen that a slight but not statistically reliable difference between the normal and the sesame oil treated series existed. That no atrophy occurred in the oil injected series, was confirmed upon histologic examination in which the cortices of these glands appeared normal in all respects. However, the weights of the adrenals of the progesterone treated animals and the control groups showed a difference which was of significance. This was further confirmed upon histological examination of the cortices of these glands. Comparison of a section of a normal gland (fig. 1) with one taken from an animal which received large amounts of progesterone (fig. 2) indicated that the zona glomerulosa as well as the zona fasiculata of the cortex was affected. In the normal gland, the cells in the zona glomerulosa appeared to be much more closely aggregated than in the experimental and the cytoplasm of the glomerulosa cells appeared to be more heavily granulated in the normal as well. No change in the capsular tissue could be noted in a comparative study of the glands in these two series. A comparison of sections through the zona reticularis and the medulla of the normal and progesterone treated animals (fig. 3, 4) revealed a distinct difference in the zona reticularis. The progesterone treated gland showed an almost entire obliteration of the normally distended sinusoids and consequently what appeared to be a crowding of cellular material in the reticularis. Figures 5 and 6 show sections through the zona fasiculata and a small portion of the zona glomerulosa of the normal and progesterone treated rats respectively. Here the cells of the zona fasiculata in the progesterone treated rat were found to be

THROUGH THE ADRENAL SHOWING THE RETICULAR LAYER AND A PORTION OF THE MEDULLA IN THE PROGESTERONE TREATED ORGAN. The sinusoidal distention is replaced by a condensation of cells in the reticularis. ×100. Fig. 5. A PORTION OF THE NORMAL ADRENAL SHOWING A PART OF THE ZONA GLOMERULOSA (UPPER RIGHT) AND THE FASICULATA. ×225. Fig. 6. A PORTION OF THE ADRENAL OF A PROGESTERONE TREATED ANIMAL. The glomerulosa is shown (upper left) and also a part of the zona fasiculata. Note the smaller cell size in the fasiculata here as compared to figure 5. ×225.

much smaller than cells of the normal gland. The nuclei of cells were closely packed. The cytoplasmic bodies were very small and, in general, the appearance of the gland through this area approximated that found following hypophysectomy. However, lipoid inclusions were still present in the adrenals of the progesterone treated rats.

The thymus showed a marked progessive atrophy in those rats which were treated with progesterone and it can be seen (table 1) that those rats which were treated with sesame oil alone also showed considerable atrophy. This latter regressive change might be explained on the basis of a reaction such as suggested by Schacher, Browne and Selye (7). Histological studies are being continued on other endocrine glands of these animals, namely the thyroid, pituitary, testes and pineal body.

DISCUSSION

The results described above show that in the male albino rat, atrophy of the cortices of the adrenal glands can be produced by the administration of large amounts of crystalline progesterone when given over a sufficient period.

These results are not surprising when the chemical similarity in the structure of progesterone and certain of the hormones of the adrenal cortex are considered. Furthermore, it has been shown (8–11) that progesterone when administered in sufficient amounts will maintain or increase the life span of various species of adrenalectomized animals. It has also been shown by a number of investigators (12–16) that pregnancy, pseudopregnancy and luteinization are factors involved in increasing the life span of adrenalectomized animals. This effect is thought to be attributed to the hormone elaborated by the corpora lutea directly or possibly indirectly through the pituitary gland.

Whether progesterone acts directly on the adrenal cortex producing an exhaustion atrophy of the 'cortin' secreting cells of the adrenal or whether the action is upon cells in the adrenal cortex which either store or actually secrete a progesterone-like substance is still a question. The degenerative changes which occur are more or less similar to those which occur when massive doses of cortin are administered. Hence, the former suggestion would seem to be the more logical. Furthermore, cellular changes of the three layers of the cortex are in line with what one might expect from cells of the 'presecretory,' the 'secretory' and the 'post-secretory' zones as postulated by Bennett (17) and others. On this basis progesterone would have to assume a cortical hormone-like activity which is contrary to the belief of Steiger and Reichstein (18). Nevertheless, the suggestion (10) that tissues of the animal might convert progesterone into the 21-oxy group of the adrenal hormone molecules is a possible solution.

It is also possible that progesterone may exert its influences on cortical atrophy through the anterior lobe of the pituitary, which may inhibit its adrenotropic activity in the presence of an excess of progesterone just as has been shown to be the action of excessive amounts of cortin (19).

This latter assumption is no more than a working hypothesis and is being investigated at this time.

SUMMARY

The purpose of this study was to determine the effects, upon the adrenal glands of male rats, of the administration of large doses of progesterone.

Daily subcutaneous injections of 4 mg. of crystalline progesterone in oil were administered for a 15-day period. Some controls were injected with sesame oil for a similar period, while others were carried along as normal controls. Two days after the last injection the body weights of all animals were recorded and the animals killed Weight records and histological studies of the adrenals were made. The following conclusions have been made.

The administration of progesterone had little or no effect upon the normal body weight increase for the 17-day period.

Atrophy of the adrenal cortex was induced by the administration of progesterone. This atrophy was especially noticeable in the fasicular and reticular zones. The cells were somewhat smaller and cytoplasmic bodies were much reduced in size. Nevertheless lipoid deposits were still in evidence in all the adrenals.

Atrophy of the thymus was induced not only by the administration of progesterone but also to some extent with sesame oil alone.

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FACTORS IN POTENCY OF THYMUS EXTRACTS

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VANCOUVER, CANADA

ROWNTREE, CLARK AND HANSON (1) reported their first extract of calves' thymi as containing 15.8 mg. reduced and oxidized sulphur compounds (calculated as glutathione) per 100 cc. They further stated their extract to be "extremely stable and entirely potent and satisfactory for injection in rats even after being kept at room temperature for 2½ to 4 years." Later attempts (2) to prepare an extract of the same stability were apparently unsuccessful.

First extracts prepared in this laboratory by Hanson's method had a sulphydryl content equivalent to 40 to 50 mg. glutathione per 100 cc. Later adopting Steinberg's method (3) the sulphydryl content was raised to around 200 mg. per 100 cc. Kept at room temperature in tightly stoppered vessels, the sulphydryl content dropped below 16 mg. per 100 cc. in 15 days. At the same temperature but with the vessels closed by loose cotton plugs, a similar drop required only 5 days. Placed in tightly stoppered vessels kept at 5°C. the extract took 80 days before it dropped below 16 mg.

From the assumption of Rowntree, Clark, Steinberg, Einhorn and Hanson (4, 5) that all biologically potent preparations of thymus extract contain iodine reducing material in excess of 15 mg. %, our extracts which were well in excess of this value should have been biologically potent.

The age limit placed upon the calves used in making thymus extract by Hanson and again by Steinberg seemed unusual and worth further consideration especially when difficulty was encountered in securing an ample supply locally of 2 to 6 weeks old calves.

Accordingly, extracts were made from thymi[1] of calves of different ages including fetal calves and the amount of iodine reducing material determined. The results have been grouped according to the ages of the calves and averages for each age and each group. These are shown in table 1.

TABLE 1. EFFECT OF AGE OF CALVES ON I.R.T. OF THYMUS EXTRACTS

Age	Fetal	Weeks			Months			Months		
	2–8 months	4	5	6	2	3	4	6	8	10
Average i.r.t. mg. %	113–183	225	192	229	150	194	249	224	168	203
	155	215			198			198		

While the iodine reducing material in extracts of calves' thymi showed considerable variation from animal to animal, the average values for different ages would seem to indicate the highest values were in calves up to 6 weeks of age, thus substantiating the earlier choice of this group by Hanson. Older calves showed slightly

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[1] Calves' thymi were supplied through the courtesy of P. Burns & Co.

lower (8%) values but still well over the level required for biologically potent extracts. It is of interest to note that the average value for the 6 to 10 months group was the same as that of the 2 to 4 months group.

The average i.r.t. computed from Steinberg's values (3) for 2 to 6 weeks old calves is 221. Our value for animals of the same age is 215. Possibly the lower value obtained by this laboratory is due to the animals being shipped 500 to 1000 miles rather than raised locally as suggested by Steinberg.

In spite of comparable i.r.t. values obtained for our extracts, we were unable by the end of the 5th generation to show any of the growth and developmental changes observed by Rowntree and his associates. Further, gross and histological examination

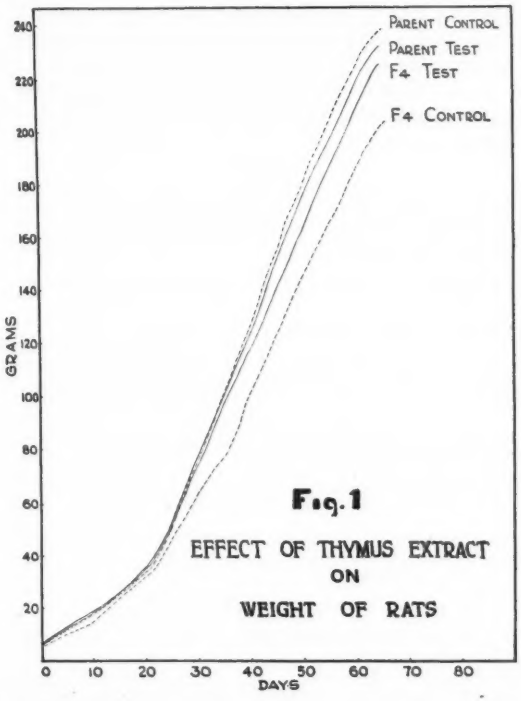

Fig. 1

EFFECT OF THYMUS EXTRACT ON WEIGHT OF RATS

of the pituitary, pineal, thyroid, parathyroids, thymus, pancreas, adrenals, ovaries and testes of both control and injected rats failed to show any difference in size, structure or manner of staining. Accordingly it seemed very doubtful whether the i.r.t. was a measure of the potency of thymus extracts.

Examination of the weight curves of the rats showed an increasing difference with succeeding generations between control and injected rats. This was due to a gradual decrease in the height of the curves of the controls while the curves for the injected animals maintained their original levels. This is shown in figure 1.

This would seem to indicate that the diet used lacked something required by the rats and that this was supplied by the daily intraperitoneal injections of the thymus extract. The diet used was "Purina Fox Pelting Chow" with occasional additions of cabbage or lettuce leaves and carrots. Food and water were given ad libitum.

The rats always appeared healthy and active. The survival rate was extremely good. The only mortalities that occurred were in an occasional litter of over 10 when one or two would be destroyed by the parent. This is in marked contrast to Rowntree's rats which showed a survival rate in the controls of only 37.8%. If the thymus extract supplied something needed to maintain weight when the survival was over 95%, it seems reasonable that it might find even greater response when the survival rate was down to 37.8%. Further, since Steinberg found that locally raised calves were in better condition and gave extracts of higher potency, one would expect rats in better condition to have thymi of greater potency. Hence these rats would be less responsive to extracts of thymi given (theory of inverse response).

In as much as our extracts were prepared by Steinberg's method and showed an i.r.t. approximating 200 mg. % yet failed to produce even a trace of the increased rates of growth and development reported by Rowntree and his associates, we feel that the condition of the thymus in the rat may be an important factor in determining the degree of response obtained from daily intraperitoneal injections of thymus extract. In this may lie the explanation for the great variance in results obtained by different workers in this field.

SUMMARY

Thymus extracts prepared from calves 2 to 4 and 6 to 10 months old showed no difference in their average content of iodine reducing material although considerable differences existed from animal to animal.

Calves 2 to 6 weeks old yielded extracts approximately 8% higher than the 2 to 10 months calves.

Fetal calves yielded extracts with the lowest values.

Iodine reducing values of thymus extracts, considered by themselves, were no indication of the potency of such extracts as judged by their effect on rates of growth and development.

Thymus injected rats maintained their weight curves while controls showed lower curves with succeeding generations.

The state of health of the rat may be a factor in the degree of response to thymus extract injections.

Histological examination of endocrine tissues of control and injected rats failed to show any difference in structure or manner of staining.

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ASSOCIATION NOTICE

ANNOUNCEMENT OF THE ANNUAL MEETING

THE TWENTY-FIFTH ANNUAL MEETING of the Association for the Study of Internal Secretions will be held in Atlantic City, New Jersey, on May 2nd and 3rd, 1941. The Seaside Hotel on the Boardwalk will be the headquarters for registration and for the scientific and business sessions.

The Chairman of the Local Committee is Dr. Matthew Molitch, 705 Pacific Avenue, Atlantic City, New Jersey.

Instructions regarding the reservation of hotel accommodations will be given to all members at a later date.

PRESENTATION OF PAPERS

Members of the Association and other investigators who wish to present papers will please note the following regulations regarding the program for the coming meetings.

1. The title of the paper, and a comprehensive abstract *in quadruplicate*, must reach the President, Dr. Elmer L. Sevringhaus, 1300 University Avenue, Madison, Wisconsin, not later than February 1, 1941.

2. Non-members who present papers will appear on the program "by invitation." Non-members are therefore requested to have their titles and abstracts introduced by a member.

3. The abstracts of papers will be considered by the program committee and the final program will be announced about March 15, 1941.

4. Papers will be limited to ten minutes for presentation and five minutes for discussion.

5. A ten-minute presentation before the Association is obviously designed for the brief and condensed discussion of new investigations, either in laboratory or clinical phases of endocrinology. Work which has previously been published or presented elsewhere, is not desired at such a meeting and consequently, it will be understood that previous publication or oral presentation before any society of national membership will be cause for omitting a paper from the program of the Association.

6. Papers reporting in final form the results discussed before the Association at its Annual Meeting should be submitted to the Editor of ENDOCRINOLOGY or the *Journal of Clinical Endocrinology*. Such submission is invited. It is to be understood, however, that acceptance of a paper for the program does not obligate either Journal to its acceptance for publication.

You are invited to bring this announcement to the attention of your colleagues and associates who have endocrine research in progress.

NOMINATION OF OFFICERS

The By-Laws of the Association provide that nominations for all elective offices shall be made by a Nominating Committee and forwarded to the Secretary at least sixty days before the annual meeting. The Nominating Committee for the current year, appointed by the President and accepted by the Council, is as follows.

Dr. Philip E. Smith, *Chairman*, College of Physicians and Surgeons, Columbia University, New York City.

Dr. J. B. Hamilton, School of Medicine, Yale University, New Haven, Connecticut.

Dr. H. B. Van Dyke, Squibb Institute for Medical Research, New Brunswick, New Jersey.

The By-Laws also provide (Article V, Section 2) that "Any member of the Association may submit nominations to the Nominating Committee for its consideration." Any nominations which members desire to make should be sent to Dr. Philip E. Smith, 630 West 168th Street, New York City, by February 1, 1941.

The terms of the following officers expire at the time of the annual meeting in Atlantic City in 1941.

President-Elect	Edgar Allen
Vice-President	K. W. Thompson
Secretary-Treasurer	E. Kost Shelton

Council Members for 3 years	Publication Board for 3 years
C. N. H. Long	F. C. Koch
Henry H. Turner	W. O. Nelson
W. O. Thompson	

THE E. R. SQUIBB & SONS AWARD

The E. R. Squibb & Sons award of $1000.00 established in 1939 and first given in 1940, is at the discretion of a special committee of five members of the Association. This award will be given to the investigator or investigators in the United States or Canada for one of the best contributions to endocrinology. Nominations for the Award may be sent to the Secretary by members of the Association, not later than January 15th of the year in which the Award is to be made. Each member has the privilege of making one nomination. Such a nomination should be accompanied by a statement of the importance of the nominee's contributions in endocrinology and by a bibliography of the nominee's most important publications in this field. Five copies should be sent to the Secretary, Dr. E. Kost Shelton, 921 Westwood Blvd., Los Angeles, California, who will transmit the copies to the members of the Committee.

Elmer L. Sevringhaus, *President*
E. Kost Shelton, *Secretary*

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1. Reichstein and Euw—*Helv. Chim. Acta* 21:1197, 1938.
2. Thorn, et al—*Am. Jl. Med. Sciences* 197:718, 1939; *Jl. Clin. Invest.* 18:449, 1939.

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